Chapter 2
Horizontal Gene Transfers with or without Cell Fusions in All Categories of the Living Matter

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Abstract This article reviews the history of widespread exchanges of genetic segments initiated over 3 billion years ago, to be part of their life style, by sphero-protoplastic cells, the ancestors of archaea, prokaryota, and eukaryota. These primordial cells shared a hostile anaerobic and overheated environment and competed for survival. "Coexist with, or subdue and conquer, expropriate its most useful possessions, or symbiose with it, your competitor" remain cellular life's basic rules. This author emphasizes the role of viruses, both in mediating cell fusions, such as the formation of the first eukaryotic cell(s) from a united crenarchaeon and prokaryota, and the transfer of host cell genes integrated into viral (phages) genomes. After rising above the Darwinian threshold, rigid rules of speciation and vertical inheritance in the three domains of life were established, but horizontal gene transfers with or without cell fusions were never abolished. The author proves with extensive, yet highly selective documentation, that not only unicellular microorganisms, but the most complex multicellular entities of the highest ranks resort to, and practice, cell fusions, and donate and accept horizontally (laterally) transferred genes. Cell fusions and horizontally exchanged genetic materials remain the fundamental attributes and inherent characteristics of the living matter, whether occurring accidentally or sought after intentionally. These events occur to cells stagnating for some 3 milliard years at a lower yet amazingly sophisticated level of evolution, and to cells achieving the highest degree of differentiation, and thus functioning in dependence on the support of a most advanced multicellular host, like those of the human brain. No living cell is completely exempt from gene drains or gene insertions.

2.1 Acquisition and Horizontal Transfer of *Vibrio cholerae* Virulence Gene

2.1.1 Hamburg 1892

In 1892 cholera struck the city of Hamburg. Unfiltered water from the river Elbe carried the pathogen into the city's drinking and cooking water supply. Allegedly "Russian immigrants brought in the disease to Prussia". Hamburg's neighboring small town Altona received its water supply from a different source. In Hamburg the number of sick afflicted with cholera reached 16,956; of these patients 8,605 died ("8,605 von 16,956 Erkrankten starben"). In Altona there were only a few cases of cholera ("Altona blieb deshalb 1892 von der Cholera weitgehend"). German steamships (Cavour,

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Elbe, Leibnitz, Normannie, Rugia) leaving Hamburg spread the disease to Antwerp, London, New Orleans, New York, and Zion. The disease was transmissible by the traveling sick or "germ carrier".

Arrived in Hamburg from Munich Herr Professor Max Josef von Pettenkofer, the pride of Germany ("der große Hygieniker"), who made Munich the cleanest city in Europe (probably in the world). Professor Pettenkofer's theory was that the multiple causations of epidemics, like the one in Hamburg, were "Miasmen", in this case organic poisons from the buried corpses of slaughtered animals in and around slaughterhouses ("Miasmen-Lehre", "Boden und Grundwasser in ihren Beziehungen zu Cholera", "Boden und sein Zusammenhang mit der Gesundheit des Menschen", "der Erreger wirkt ausschliesslich durch verseuchtes Grundwasser"). In order to prove that no special bacteria caused cholera, even before the Hamburg outbreak, Professor Pettenkofer and two members of his staff (two assistants) swallowed the liquid contents of a vial containing cultured cholera vibrio bacteria. On his request, the vial was sent to Professor Pettenkofer by Professor Koch. The three self-infected volunteers became ill with diarrhoea but survived. They were claiming gloriously thereafter that it was not the cholera vibrio that caused cholera and/or the deadly epidemic now in Hamburg.

Arrived in Hamburg Herr Professor Robert Koch. He saw the vibrio under the microscope in the intestinal tract of the dead in 1883 in Alexandria, Egypt (like Filippo Pacini in 1854 in Firenze), but could not culture it. In Egypt, the cholera epidemic in 1883 claimed over 58,000 (100,000?) lives. It was later in India in the same year that Koch produced pure cultures of *V. cholerae* "swarming on gelatine plates." The pathogen was "ein kurzes, kommaähnlich gekrümmtes Bakterium." "Die Identifizierung des Cholera Erregers" and the established fact ". . .daß Koch den Choleraerreger gefunden hatte" followed. In Hamburg Professor Koch educated the inhabitants of the city that drinking water ("Übertragung von Cholera durch Trinkwasser") carried the germ of the disease, advocated strict isolation of the sick, introduced disinfection ("Desinfektionskolonne mit Chlorkalkkarren während der Cholera-Epidemie in Hamburg 1892"), quarantined the ports of the city, and demanded that the city's water supply be sand-filtered. The epidemic was halted[1].

2.1.2 The Vibrio and the Disease

In their aquatic habitat, *Vibrionaceae* enzymatically degrade chitins of crab and crustacean shells [1]. Of the 205 *Vibrio cholerae* serogroups, only O1 and O139 cause cholera epidemics; other members of the group may cause diarrhoeal illness, but not cholera [2, 3].

The non-choleragenic serogroup members produce vibrio cytolysins (VCC) and hemolysins. Anion channels opened up by VCC in the membranes of enterocytes trigger an outpouring of chloride, sodium and water; such efflux results in the watery diarrhoea and dehydration of the host [4]. The infamous El Tor biotype belongs to the O1 serogroup. The El Tor vibrios carry multiple antibiotic-resistance gene clusters within transposon-like structures (cassettes; constins, operons). The genes confer resistance of the vibrio to streptomycin, sulfamethoxazole, trimethoprim, and chloramphenicol (STX). The STX genes reside within a large integrating conjugative element (ICE). The transfer of these excised gene clusters occurs by conjugation and integration (not by phage-mediation) [5–7]. In two chromosomes (one large, one small) vibrios carry 142 and 42 single copy genes, and gene clusters for virulence and the inserted genomes of its prophages, respectively. Smaller genetic entities in the cytoplasms are the plasmids, frequently the vectors of horizontal gene transfers ("rampant" such activity) [8].

[1]Max von Pettenkofer Institut für Hygiene und Mikrobiologie. Pettenkoferstrasse 9a, München D-80336; Robert Koch Institute, Nordufer 20, Berlin, D-13353; Richard J. Evans: Death in Hamburg. Oxford: Clarendon Press, 1987 xxii 676 pp.

The pathogenic cholera vibrios (*V. cholerae* or *V. mimicus*) replicate in the small intestine, and release their AB-type toxin, thus inducing the profuse secretory diarrhoea leading to electrolyte losses, profound dehydration, lipopolysaccharide (LPS) endotoxin shock [9] and death. Cell surface gangliosides bind the pentameric subunit CTB. The cholera toxin CTA after transgressing the plasma membrane of the host cell is taken up by the endoplasmic reticulum (ER). In the ER, the monomeric subunit A1 chain is unfolded and directed to pass through the protein-conducting channel into the cytosol. Escaping degradation by proteasomes, the toxin refolds and acts [10]. The innate proinflammatory responses induced by the cholera toxins consist of the activation of the Akt cascade resulting in the overproduction of interleukin-1α (IL-1α), IL-6, and tumor necrosis factor-α (TNF-α) and the translocation from cytoplasm to nucleus of nuclear factor kappa B lineage lymphocyte (NF-κB) [11] The major immunosuppressive effect of the cholera toxin is inhibition of IL-12 production in dendritic cells (DCs), thus it is the abrogation of a Th1-type immune response in the gut. This is further achieved by the inhibition of interferon (IFN) regulatory factor-8 (IRF8), thus plasmacytoid DCs remain undifferentiated non-IFN producers. Even if IL-12 were produced, CTAB inhibit the expression of IL-12R (beta receptors β1 and β2). Immune T cells, if generated, do not release interferon-γ (IFN-γ). The immunoglobulin response is mainly that of IgE; thus further toxin outpour is not neutralized, but it is reacted to with anaphylactoid events culminating in shock [12]. The beta subunit of CTB induces a tolerogenic response mediated by both antigen-specific $FoxP3^+$ regulatory T cells (T_{reg} cells) and by transforming growth factor-beta- (TGF-ß) and IL-10-producing T cells [13]. The major *V. cholerae* toxins are the cholera toxin (CTAB), neuraminidase, chitinase and LPS endotoxin. In *V. cholerae* antigen- (toxin-) exposed mice, peritoneal macrophages died apoptotic deaths [14].

Overcoming the immunosuppressive and tolerogenic effects of CT, the host mobilizes immune reactions against the vibrio and its toxins. Patients recovering from cholera withstand a second exposure to *V. cholerae*. These individuals possess memory B and T cells in Peyers' patches and in the circulating blood. Antibodies reacting to re-exposure are of the IgA and IgG classes and react with LPS and CTAB [15]. During the acute disease and in convalescence, patients begin circulating $CD4^+$ and $CD8^+$ gut-homing T, and $CD19^+$ B cells, Upon stimulation with vibrio cell membrane or TCP (toxin co-regulated pilus) antigens, these cells respond by clonal expansion and release of INF-γ (for Th1-type immunity) and/or IL-13 (for Th2-type immunity) [16]. These immune reactions serve as reasoning for the development of preventive cholera vaccines. The vaccines offer good to partial protection, but their immune efficacy weakens without re-vaccination [17–20]. The Peru-15 (CholeraGarde; AVANT) live-attenuated oral vaccine was safely and effectively administered to Bangladesh infants and toddlers [21–23]. The College of Medicine, University of Central Florida is developing a united malaria-cholera vaccine [24].

Bicarbonates stimulate the ToxT regulatory protein, thus the transcription of CT and TCP. The contents of the small intestine are bicarbonate-rich. Ethoxyzolamide inhibits carbonic anhydrase and negates the stimulatory effect of bicarbonates on ToxT [25]. Resveratrol (3,4′5-trihydroxystilbene) inhibited cholera toxin-induced damage in Vero cells. The toxin was precipitated and its endocytosis was inhibited. In the cells, the toxin's effect on cyclic adenosine mono- or diphosphates (AMP/ADP), such as AMP accumulation and ADP-ribosyltransferase activity, were suppressed [26]. The expression of cholera toxin in the cell is regulated by the transcriptional cascade of ToxT. Cis-palmitoleic acid reduces the expression of both virulence factors of the cholera toxin (CT) and prevents ToxT from binding DNA [27].

There exist natural vibrio isolates that harbor a provirus CTXφ, which does not possess the *ctxA* and *ctxB* genes. In addition, these prophages lack the upstream control region normally located 5′ of *ctxA*, and the promoter region and coding sequences of *ctxB*. In these phages, the ancestral precursor of the CTXφ phage was found still in existence. Therefore the *ctxAB* genes do not behave as vertically transmitted genuine phage genes; they are acquired genes and as such they were gained through horizontal transfer [28, 29]. The cluster of these genes and their regulatory sequences must have been acquired simultaneously. Horizontal transfer of the CTXφ genes must have occurred repeatedly.

The CTXφ phage enters the vibrio through its type IV pilus, TCP (*vide supra*). The cluster of vibrio genes encoding the pilus can be transmitted between vibrios by unknown mechanisms [30]. The OrfU (open reading frame) protein binds CTXφ phage particles to TCP, as their attachment receptor. The pili serve the bacterium as essential colonization factors in the human small intestine. The bacterial flagellae penetrate the mucosal layers of the small intestine. Flagella loss (Fgl^{-}) releases the anti-sigma and alternative sigma factors and these repress quorum sensing regulators with the release of virulence factors [31]. Excessive colonization frequently assumes the form of biofilms [32, 33]. The CTXφ phages infecting classical and El Tor vibrios are distinct, but diverged from a common ancestor. It is the *orfU* and *zot* (zona occludens toxin) genes of these CTXφ lineages that show this divergence. It was not the ancestral CTXφ that infected an ancestral vibrio, but rather it was its two lineages, which infected separately the classical and El Tor vibrios. Phage CTXφ of *Vibrio cholerae* could infect *Vibrio mimicus* and transfer horizontally the cluster of these genes with their regulatory sequences into its new host. *V. cholerae* and *V. mimicus* diverged from a common ancestor, and their acquisition of CTXφ occurred after their divergence. First *V. cholerae* might have been infected, and from there *ctxAB* operon was horizontally transferred by CTXφ into *V. mimicus* [28].

The highly epidemic new strain of *V. cholerae,* O139 Bengal, emerged in 1992–1993. It replaced the pre-existing O1 serogroup El Tor strain. In 1994, the O1 serogroup El Tor strain recurred and re-occupied its territory. Later in 1996, O139 re-emerged and thereafter co-existed with O1 El Tor *V. cholerae* [34]. The new O139 *V. cholerae* strain derived from an ancestral El Tor vibrio with preservation of its virulence factors, but with new and different serotype. *V. cholerae* O139 exhibits the insertion of a large new genomic region foreign to the pre-existing O1 El Tor strains, while the O139 vibrio suffered a deletion all of its O1 antigen-specific gene cluster. The O-antigen biosynthesis gene cluster occupies the *wbf* (wild-type biofilm) region in the genome of the vibrio O139. The O139 LPS is antigenically different from that of the O1 El Tor vibrio. Patients recovering from O1 El Tor vibrio-caused cholera remain susceptible to infection with the vibrio O139. A large portion (22-kb) of DNA strands of the regulator of biofilm region (*wbf; rfb*) was deleted in the O139 vibrio. This deleted region is replaced by a new fully sequenced 35-kb *wbf* region encoding the O139 antigen. It is also possible that the new DNA segment was not transposed from an outside source, but that it originated by homologous recombination events within the ancestral El Tor vibrio residing in biofilm colonies on chitin surfaces (chitin skeletons of crustaceans) in aquatic reservoirs, where resistance acquired against bacteriophages were the driving force. The emergence of O139 vibrios in the intestinal tract of individuals who survived prior exposure to O1 El Tor *V. cholerae* indicates that ineffective host immune reactions might be another driving force of the transformation [35–37]. Chitin-induced natural transformation resulting in O1 conversion to a different serogroup (non-139 and O139) occurred experimentally. It is the O1 recipient (the El Tor vibrio) that acquires a new O139 LPS-antigen-encoding cassette. These cassettes are incorporated into the recipient genomes by homologous recombination. The exact mechanism of such large gene cluster (operons) transfers from the transformed vibrios into not yet transformed vibrios remains unclear. Under consideration are conjugative plasmids as vectors, transducing but as yet undiscovered phages, and other as yet unrecognized mechanisms.

In addition to phage-mediated CTXAB, cholera vibrios express the protein synthesis inhibitor cholix toxin [38] and hemolysins, which render bilayered plasma membranes of eukaryotic cells non-selectively and indiscriminately permeable [39]. The multifunctional autoprocessing repeats-in toxin (MARTX) destroys the actin cytoskeleton in eukaryotic cells [40, 41]. Some of the hemolysin genes (*hly* :Hly) encoding these toxins may reside in the virulence islands of the vibrios, but they appear to be genuine vertically transferred bacterial genes.

Enterotoxigenic *Escherichia coli* (ETEC) produces an enzymatically active A subunit toxin and a receptor-binding pentamer B subunit toxin. Its Longus pilus induces self-aggregation and adherence of the bacteria to intestinal epithelial cells. The ETEC lytic phage enters the bacterium through a colonization factor pilus [42–45]. Phages lytic to ETEC cells are well known, but toxin-encoding

phages remain elusive. The C57 pilus is the entry site of most ETEC phages. The porcine ETEC phage phiEcoM-GJ1 is a recombinant of a Myoviridae, a Podoviridae and a Siphoviridae bacteriophage with Myoviridae outside morphology (icosahedral head, contractile tail with fibers). There are no toxin-encoding genes in the genome of this lytic phage [46, 47]. Lambdoid bacteriophages possess and spread Shiga toxin (*stx*) genes in populations of *E. coli* bacteria: for example, Shiga toxin- (Stx-) producing *E. coli* (STEC), such as *E. coli* strain O157:H7 of cattle origin [48]. From the point of view of the bacterium, the toxin promotes its colonization in the gut of cattle (or human patients) and protects it from bactivorous protozoa, like *Tetrahymena pyriformis* in the colon of the cattle [49]. Through unknown ancient mechanisms, Shiga toxin-encoding bacteriophage 933W acquired and operates a eukaryotic-like ATP-binding and phosphotransfering serine/threonine tyrosine protein kinase-encoding gene (*stk*) [50].

The High Pathogenicity Islands (HPI) may travel with excessive speed and long distances in between human communities and from hospitals to hospitals infecting strains of *Enterobacteriaceae* through horizontal routes of transfer. The *Enterobacter hormaechei* outbreak in the Netherlands occurred by transfer of a new variant HPI to *E. coli* and *K. pneumoniae.* Both the genomic islands (GI) containing the virulence genes (named in the article) and the genomic modules (GM), five GMs listed one by one in the article, were transferred. The new HPI contained integration sites to a mobile DNA element. The mobile DNA element was able to excise, circularize and insert the HPI at multiple sites: multiple combinatorial transfers of both GI and GM occurred [51]. In the USA, uropathogenic *E. coli*, and in France, *Salmonella typhimurium*, enterohemorrhagic and uropathogenic *E. coli* are suspect to have acquired through horizontal transfer HPIs. This subject matter will be returned to later in the discussion of horizontal gene transfers across prokaryotic lineages through rapid pathway evolution against the background of the evolution of prokaryotic genomes (*vide infra*, in The Darwinian threshold).

2.1.3 The Phage and Its Genome

One of the filamentous phages of *V. cholerae* is CTXphi (CTXφ). This phage carries the cholera toxin (CT) genes *ctxAB*. The 6.9-kb genome of CTXφ integrates into the genome of its host, the vibrio (*V. cholerae*). Replication sequences (RS) encode the enzymes needed for the integration of the viral genome into the host genome. The genome of the classical El Tor vibrio does not offer an integration site to the phage. In this host, the CTXφ phage exists as an extrachromosomal circular DNA plasmid. Several genuine phage genes encode the structural proteins of the virus and their assembly into a particle (among them open reading frame U, *orfU*, and zona occludens toxin, *zot*). The non-integrated plasmid is the replicative form (RF) of the phage and produces an abundance of viral particles. These plasmids lack ctxAB and ToxR binding sites, but possess a *zot* sequence, which is different from the *zot* gene of the pathogenic *ctxAB*-containing integrated phage. These plasmids must have derived from the ancestral preCTXφ phages. Not the host cell, but *ctxA* and *ctxB* gene-carrier phages encode the cholera toxins CTXAB. The GC content (34–37%) of the three *ctxAB* genes ($CTX^{ET\varphi}$, $CTX^{class\varphi}$, $CTX^{calc\varphi}$) significantly differ from that of the genuine phage genes. The *ctxAB* phage genes must have evolved differently from the genuine phage genes. There is no similarity, indeed there is a lack of congruence, between the genuine vibrio gene *mdh* (encoding malate dehydrogenase) and the two phage genes *orfU* and *zot* (required for coat proteins and their assembly). The *mdh* genes are identical in classical and El Tor epidemic *V. cholerae* isolates, whereas the CTXφ genes are widely divergent. Thus, CTXφ genes behave like mobile genetic elements [28]. Indeed, *ctxB* contains a Mariner-based transposon [52]. The pathogenicity genes of *V. cholerae* operate from pathogenicity islands of the genome. Vibrio pathogenicity island-2 (VPI-2) encodes integrase, recombinase, a restriction modification system, Mu phage-like proteins, neuraminidase and glycosylhydrolase, and other sialic acid

metabolizing enzymes; these enzymes expose GM1 gangliosides, which serve as receptors for cholera toxin. El Tor and O139 cholera vibrios operate the vibrio seventh pandemic island (VSP). These islands can excise and re-insert themselves from and to the vibrios' genome [53]: ready for horizontal transfer and insertion. These horizontally transferred and genomically inserted virulence genes have been acquired "recently" and repeatedly, are clustered in several chromosomal regions and derived from an unknown original source [54].

The core region of the CTXφ prophage encodes CT, structural proteins for its morphogenesis and the repeat sequence region-2 (RS2) for regulation of its replication and integration. Another RS element (RS1) is inserted next to and flanking the integrated phage genome. This RS1 segment contains all open reading frames of RS2 and the truncated gene *rstC*. The RS1 element can exist in a single-stranded circularized form and in an excised double-stranded replicative form (RF) and as such it may enter host cell genomes horizontally [55–57]. *V. cholerae* Mozambique 2004 strains carry a tandem repeat of the CTXφ prophage integrated both into its small and large chromosomes; RS1 element *cla, env*, and CTX elements *env* and *rstR* (env) are integrated in the vibrio's large chromosome [58]. It was unexpected that the Mozambique 2004 cholera epidemic was caused by the O1 serotype El Tor vibrio infected not with the $CTX^{ET\varphi}$, but with the $CTX^{class\varphi}$ phage; another Indian (Kolkota, India) El Tor vibrio strain is infected with the $CTX^{class\varphi}$ phage. The difference was the absence of the El Tor-specific free RS1 element of the prophage in the Mozambique vibrio, while the Indian vibrio strain possessed this element [59].

The O139 strains isolated in 1992–1993 harbored two copies $CTX^{ET\varphi}$ connected to an RS1 element (*vide supra*). The O139 strains spreading upward from Bangladesh through the Ganges delta and to the Indian continent held three copies of the CTX prophage in tandem arrangement. One of these prophages is different from the $CTX^{ET\varphi}$ in its *rstR* gene, which encodes the repressor protein of CTXφ; it is referred to as the $CTX^{cal\varphi}$ (Calcutta) prophage. Thereafter O139 isolates form different ribotypes and undergo further genetic diversity and genetic reassortments [36, 60, 61] The antibiotic resistance (SXT, *vide supra*) genes in O139 were acquired by 62-kb self-transmissible transposon-like elements; these genes are also transmissible by conjugation (*vide supra*). Indeed, by the transfers selective advantage (phage; host immunity and antibiotic resistance) was conferred to the recipients [37]. Vibriophage-mediated CT gene horizontal transfers between donor O1 El Tor to recipient non-O1/O139 vibrios occurred recently in California coastal waters [62].

Repeat sequences (RS1, 2) flank the integrated CTXφ gene (*vide supra*). The ds replicative form (RF) of the RS1 element was marked with a kanamycin resistance (Kmr) marker (pRS1-Km). The pRS1-KM construct in O1 vibrios acted like the filamentous phage RS1-Kmφ. All classical, El Tor and O139 vibrios are susceptible to this phage; nontoxigenic (CTX^{-}) vibrios encoding TCP are the most susceptible. Using the integration sequence *attRS* (attachment), the RS1φ genomes also integrate into vibrio chromosomes. Only fused genomes of RS1-KmφCTXφ generated extracellular phage particles. Thus, these phages are transmitted vertically in the host vibrios, but are able to propagate horizontally as well. The cholera toxin-encoding, *ctxAB* gene-carrier CTXφ integrates into host cell chromosome at attachment site *attRS* due to the RS2 region within the CTXφ genome. The RS region encodes regulatory, replicative, and integrative functions of the CTXφ phage. The integrated toxigenic CTXφ genomes are flanked by the RS1 element possessing ORFs *rstA, B, R*; RS1 expresses one additional ORF, *rstC*. The RS1 excised from the chromosome may act as a filamentous phage particle, but without containing CTXφ genomic elements. In contrast, in CTXφ virions the RS1 gene *rstC* is replaced by the core genes of CTXφ. The RS1 genome encodes the RstR repressor protein. The CTXφ genome carries the gene for the RS2-encoded repressor protein. Phage-induced repressor proteins in many phage-carrier bacterial genera maintain the lysogenic state. By the acquisition of its own new core genes, CTXφ separated from its ancestor, the RS1 element, and became a new phage. Its RS1 ancestry is further evidenced by the ability of CTXφ to integrate into its host cells' genome [63a].

The RS1φ phage remains a satellite phage in the genome of the vibrio. CTX-negative vibrios do not replicate the RS1φ phage. The exception is the non-toxigenic *V. cholerae* strain 55V71. Thus, the 55V71 genes are essential for the replication of the RS1φ phage. *V. cholerae* strain55V71 harbors another filamentous phage. The 7.5-kb ssDNA of the 55V71 gene cluster acts as if it were the genome of another filamentous phage, KSF-1φ. This phage enters the vibrio through its mannose-sensitive hemagglutinin pilus. The presence of this phage genome in the vibrio promotes full maturation and horizontal transfer from vibrio-to-vibrio of RS-1φ phage particles. Thus, there is a close cooperation of at least three phages (CTXφ, RS-1φ, KSF-1φ) in the transformation of non-toxigenic to toxigenic strains of *V. cholerae* [56, 57, 63a].

Not one or two, but several filamentous phages cooperate to encode the cholera toxin: the well known filamentous lysogenic phage CTXφ, the replicase-encoding toxin-linked cryptic element, TLC, whose genome consists of the morphogenesis-encoding satellite filamentous phage, fs2φ, and the phage genomes encoding infectious TLC-Knφ phage particles. The recombination sequence encoded by the TLC-Knφ phage is used by phage CTXφ to integrate its genome into the vibrio's genome. The ssDNA genome of phage fs2 is the circularized variant of the TLC genome. In order to achieve the acquisition of toxigenicity by a pandemic *V. cholerae*, two satellite filamentous phages (TLCφ, RS1φ), three helper filamentous phages (fs2φ, CTXφ, KSFφ) and two type IV pilus-based phage receptors (MSHA, TCP) have to establish molecular interactions. (KnR = kanamycin resistance; MSHA = mannose-sensitive hemagglutinin; RS = repeat sequence region; KSF = Kamruzzaman, Sack, Faruque; TCP = toxin-coregulated pilus; type IV cholera vibrio pilus) [63b]. It is now well understood how filamentous cholera vibrio phages insert the toxin gene(s) into the bacterial host's genome and how they encode the toxin from the genome of the host bacterium. It remains to achieve a grasp of the origin of the toxin genes that were horizontally transferred originally into the phages' genome from an unknown source (a vibrio ancestor that acquired the toxin gene(s) from a mysterious eukaryotic host still in hiding).

2.1.4 The Hiding Place of the Original Cholera Virulence Genes

The planktonic marine and estuarine aquatic environments vibrios inhabit, is close to be saturated with archaea, prokaryotes and protozoa and their viruses. Just the tailed phage particles in the environment are estimated to number 10^7/ml, all phage particles 2.5×10^8/ml, and in total on the planet 10^{31} particles. The estimate was offered that 10^{25} phage infections occur/sec worldwide; counting retroactively for 3 billion years, the number of phage infections that had taken place on Earth is incalculable (cited in [64]). Metagenomic characterization of the viral flora is discovering innumerable new members of viral genera. Metagenomic analysis (pan-viral microarrays, polymerase gene sequencing, high throughput sequencing) of seawater and reclaimed water samples for "marine viromes," "marine phage genomics" and "global virospheres" revealed an abundance of free bacterial genes, bacteriophages, pathogenic plant viruses, eukaryotic nucleocytoplasmic large dsDNA viruses and picorna-like eukaryotic RNA viruses [65–71]. This is the environment in which *V. cholerae* and its phage CTXφ acquired the CT genes *ctxAB* from an unknown external source. What is the biological function of the gene product proteins, the CTAB, in the vibrio? It is in the virulence island where the genes *tcp* encoding the pilus TCP reside. It was by sequential acquisition that the predecessor of *V. cholerae* acquired then the *tcp* and *ctxAB* genes [64]. However, the biochemical function of the CTAB in the human small intestine (*vide supra*) indicates that these molecules fit best into eukaryotic biological systems, thus must have originated from a eukaryote host.

The non-choleragenic serogroup vibrios can cause watery diarrhoea and enterocolitis (*vide supra*). Were the non-choleragenic vibrios exposed to CTXφ and related phages, but successfully defended

themselves by the prokaryotic RNA interference-mediated defense system? This system consists of the clustered regularly interspaced short palindromic repeats with closely associated genes (CRISPR; CASS). If this antiviral mechanism worked faultlessly, phages and viruses would have been extinguished from the rest of evolution. However, viruses are able to circumvent the CRISPR/CASS barrier [72]. The system was found to be installed in *V. cholerae* O395: this vibrio is capable of mediating an RNAi interference pathway [73]. The a-virulent *V. cholerae* strain ATCC14033 (American Type Culture Collection) isolated in 1910 is considered to be the predecessor of the highly pathogenic El Tor vibrio [74]. Its pathogenicity island is devoid of the *toxP* and *tcpP/tcpH* (hemolytic) genes, yet it could not protect itself from transformation to the El Tor vibrio class as it has accepted the phage-mediated horizontal insertion of the *ctxAB* operon. Where did CTXφ and related vibrio phages acquire the *ctxAB* operon from?

Vibrios populate the intestinal tracts of sea birds, sea mammals [75], fishes, crustaceans (*vide infra*), sea horses [76], mollusks, oysters and coral [77]. These bacteria- and virus-infected (*V. alginolyticus, V. parahaemolyticus, Listeria monocytogenes*, hemorrhagic septicemia virus) hosts mobilize innate and adaptive immune reactions. The title of this report does not reflect to its deep penetration into basic immunology [78]. Crab hemocytes and some parenchymal cells express antibacterial peptides (crustin; anti-lipopolysaccharide factor) in response to *V. alginolyticus* infection [79]; through these responses, some of the afflicted hosts survive as vibrio-carriers. Halophilic vibrios are rich in substances (neuraminidase, hemolysin, permeability factor, lethal toxin) which kill mammalian cells [80]. The toxic *gyr* (gyrase) gene was present, while the CT *ctx* genes were so far not identified in halophilic vibrios [81] The vibrios notoriously carry phages and release plasmids. For example, the halophilic vibrio, *V. alginolyticus* possesses covalently closed circular plasmids 6,075 bp in length with 42% GC content and seven ORFs encoding over one hundred amino acid length sequences. These encoded proteins are relaxases, replicases and mobilization proteins (MobC) [82]. The vibrios deriving from these hosts may go through the human food chain. The vibrios are not intracellular bacteria, but they adhere to cell surfaces. Could the vibrios pick up eukaryotic genes? Could the phages or plasmids of the vibrios incorporate such eukaryotic genes deriving from their hosts? Of these possibilities, the last step is the most likely: that is, vibrios co-express their phage receptors and exchange their phages and plasmids. This imaginary chain of events presumes the acquisition of eukaryotic genes by a vibrio and its phage in their natural habitat (*vide infra*).

The attachment of vibrios to crustaceans, dead or alive, is mediated by the bacterial chitinases. Vibrios form biofilms on the dead crustaceans' chitin shells. Numerous viruses infect crustacean colonies (baculovirus, hypodermal and hematopoietic necrosis virus, hepatopancreatic parvovirus, white spot syndrome virus, myonecrosis virus, yellow head nidovirus, Taura syndrome virus, nodavirus, gill-associated virus, loose shell syndrome virus, spawner-isolated mortality virus) [83, 84], and others. In a small segment of the oceanic viral flora, shrimp and prawn viruses abound. A brief list of these viruses is provided with references. No implications are offered that any of these viruses could be picked up by vibrios and from them by vibrio phages. Human pathogenicity, if any, would come through the food chain (the List). Such viral infections are devastating to crustacean colonies, however crustacean hosts defend themselves by all means of innate immunity (anti-lipopolysaccharide factor, lectins, RNAi, CRISPR/CASS) and some manage to survive [85–87]. Molting copepods and nauplii of the zooplankton are able to spread some of these viruses [88]. Vibrios colonize the chitinous exoskeletons of certain copepods [89]. However, no vibrios were as yet isolated that would have picked up a crustacean virus; copepods transfer some of these viruses from crustacean to crustacean hosts, but not to vibrios. The so far sequenced genomes of crustacean viruses were devoid of *ctxAB*-like segments. Thus, the derivation of the *ctxAB* eukaryotic genes and the mode of their transfer to the CTXφ and related vibrio phages remains an unresolved problem.

The common ancestor of extant vibrios emerged in the sea about 600 million years ago [90]. Crustaceans and fish harbor pathogenic and non-pathogenic vibrio species [91–94]. Vibriosis with *V. splendidus* of larval turbots carries significant mortality. *Vibrio harveyi* causes "luminous vibriosis"

of shrimp and expresses hemolysin gene(s) (*vhh*). Shrimps or lobsters (*Homarus* sp.) with hemorrhagic and ulcerative enteritis carry vibrio sp. without proven etiologic relationship to the disease in their intestinal tract [95–97]. Is it far-fetched to consider the acquisition of the disease-causing genes from these hosts by the vibrios and from the vibrios by their phages, which promiscuously infect other species of vibrios including vibrio species with human pathogenicity?

Zooplankton blooms precede the outbreaks of cholera epidemics [98]. Is there a so far hidden niche for the *ctxAB* operon in zooplanktons or crustaceans (*vide infra*)? An imaginary sequence of events is envisioned, in which a toxin-producer marine/aquatic-estuarian eukaryotic host is infected by a phage-carrier bacterium: a vibrio. The bacterium acquires the toxin gene from its host and gains invasiveness and proliferative advantage. The lysogenic phage integrated its genome into the genome of its host bacterium, the vibrio. The phage excises its genome from the bacterial genome. The genuine phage genes in the excised genome include full or close to full sequences of the toxin gene(s). At burst, the liberated phage particles find another host bacterium, which they invade and into whose genome they integrate their phage genome. That newly infected bacterium has pathogenicity (virulence) islands towards mammalian (including human) hosts. The integrated toxin gene-carrier phage genome encodes the toxin. The bacterium gains invasiveness and proliferative advantages in his infected host due to the expression of the toxin proteins. The infected host dies due to the pathophysiological effects of the toxin (*vide infra*).

An abbreviated listing of Shrimp and Prawn viruses: Baculovirus [99a]; Dicistroviruses [100]; Hepatopancreatic parvovirus [99b, 101]; Taura syndrome virus [102–104]; Yellow head nidovirus [105]; Gill-associated and yellow head okavirus [106]; Whispovirus. White spot syndrome virus [107–110]; Loose shell syndrome virus [111]; Crustacean antiviral immunity [112].

2.2 Mimivirus and Its Companions

2.2.1 Viruses and Toxins of Blooming Dinoflagellates

Chloroplasts derive from cyanobacteria; the gene orders preserved in chloroplasts prove their cyanobacterial origin [113]. Extreme intraphylum diversity characterizes cyanobacterial aminoacyl-tRNA synthetases. Horizontal gene insertions, deletions and gene duplications created inconsistencies in the evolutionary course of these enzymes, the intraphylum diversity of aminoacyl-tRNA synthetases [114]. Filamentous freshwater cyanobacteria and the dinoflagellates *Alexandrium* spp. produce paralytic shellfish poisoning toxins, but encoded from different gene clusters [115a]. The 26 putative saxitoxin genes (stxA to stxZ : STX) were identified in toxic cyanobacteria (not to be mistaken for the STX antibiotic resistance gene-product proteins). Some of these genes (17 of them) are of cyanobacterial origin. Other genes in the cluster originate from different other cyanobacteria species, a halodurans archaea, the delta-proteobacterium *Myxococcus xanthus*, and the actinobacterium, *Frankia.* A methyltransferase may be of the dinoflagellate *Alexandrium tamarense* derivation. The non-cyanobacterial genes were acquired through horizontal transfers. Did the dinoflagellate *Alexandrium* receive cyanobacterial toxin genes in exchange? The abundant cyanobacterial phages (myo-, podo-, and siphoviruses) are diligent vectors of host cell genes. For example, the bacterial photosystem-I and II genes are propagated by cyanophages. A unique, not T4-like myovirus cyanophage infects the blooming and toxic cyanobacterium *Microcystis aeruginosa*. The *M. aeruginosa* toxin induces hepatocellular carcinoma in experimental animals. This phage possesses a large 162, 109 bp genome containing 184 protein-coding genes. While several host bacterial genes have been incorporated into the genome of this phage, the bacterial toxin-encoding genes could not be found among these horizontally trsnsferred genes [115b]. Were the cyanobacterium a human pathogen, and the phage a carrier of the toxin-encoding genes, a system analogous to that of *V. cholerae* would have been identified.

The chromalveolate diatoms possess genes of red algal origin; endosymbiotic red algae delivered these genes into the nuclei of diatoms [116].

Bacteria attached to the surface of dinoflagellate cells (*dinos*, Greek: rotation, eddy) were not transformed into toxin-producers [117] The blooming *Raphidophyceae* family member microalga *Heterosigma akashiwo* causes red tides. Chinook salmons and amberjacks die in the red tides. The large DNA *H. akashiwo* virus (HaV) kills the microalga and as a lytic algicidal virus is able to clear red tides, not in natural, but at least in experimental conditions [118]. The hemolytic and cytotoxic karlotoxin and ichthyotoxin from the marine dinoflagellate *Karlodinium veneficum* kill fish [119]. Other dinoflagellate toxins (pectenotoxin, yessotoxin, okadaic acid toxins) are hepatotoxic and cardiotoxic in experimentally exposed mice [120]. Yessotoxin poisons bivalve mollusks; when injected intraperitoneally into mice, it inhibits phagocytosis of *Candida albicans* by macrophages [121]. *Gambierdiscus* spp. dinoflagellate microalgae produce ciguatoxins (abbreviated as CTX: not to be mistaken for cholera toxin) [122]. The alga *Karenia brevis* in the Gulf of Mexico, produces brevetoxin and its antagonist brevenal; lower water salinity favors more toxin and less antitoxin production [123].

Blooming phyto- and zooplanktons release the toxins that are frequently lethal to crustaceans, shellfish, and higher metazoans, including human patients, if these toxins enter the food chain. The same dinoflagellates, algae, amoebas and diatomes carry a large number of bacterial, fungal and protozoal symbionts (including vibrios, like *V. alginolyticus*), and viruses, both lytic and symbiotic, either attached extracellularly, or invading intracellularly [124].

Juvenile coral cells take up for intracellular endosymbiosis zooxanthellae *Symbiodinium* algae, and thus gain photosynthetically produced carbon-rich nutrients. Free-living symbiodinium cells do not, but these cells in symbiosis with coral cells do activate their H^+-ATPase. This 105 kDa protein works as a proton pump, dehydrates bicarbonates by carbonic anhydrase and liberates inorganic carbon. The chloroplast-encoded ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisCO) enzymes provide the fixed carbon both for the symbiont and its host [125]. Both the nuclear and mitochondrial genomes of the coral cell and the genome of the symbiont were sequenced. The symbiont resides in the gastrodermal cells of the coral and provides fixed carbon to these cells. The symbiotic relationship depends on the acceptance of symbiodinium sub-clade by the haplotype coral [126–130]. Entry of the symbiont in a stealth manner leaves the host cell's transcriptome undisturbed. Juvenile corals are rapidly dominated by the symbiont, whereas adult or parental coral cells are less tolerant toward the symbiont. Compatible symbionts are accepted without a reaction, but incompatible symbionts massively excite the recipient's transcriptosomes [131]; The expression of metabolic genes (carbohydrate and lipid metabolism, transmembrane ion transport) is intensified in corals possessing symbiont algae [132]. Possession of the symbiont keeps the coral from senescence in that its telomere length is preserved by continuous telomerase activity [133]. Rejection of the unacceptable symbiont is carried out by caspase-activated apoptosis of the algal cells [134]. Mature corals may lose the symbionts during "bleaching" events. Loss of the symbionts frequently leads to the demise of coral colonies. Corals retaining the symbionts during the bleaching event survive [135]. Coral colonies attacked by *Vibrio coralliilyticus* undergo bleaching and die. The target of the vibrio protease is more the intracellular symbiont, than the coral tissue [136]. It is most peculiar that a coral-pathogenic vibrio (*V. coralliilyticus*) in the Mediterranean sea would be multiple antibiotics-resistant [137]. Antibiotic resistance genes must have evolved under natural circumstances predating with millions of years the discovery and use of penicillin: "the soil antibiotic resistomes." A transposon-induced mutant of *V. coralliilyticus* lost its flagellum, and with it its pathogenicity [138]. It was not reported if this vibrio harbored phages. A 81 pages, 451 references outstanding article on the biodiversity of vibrios managed not even to mention vibrio phages [139]. *V. coralliilyticus* infections of corals induce mass mortality in the warm Mediterranean sea [140]. Corals defend themselves against bacterial and fungal pathogens by mobilizing amoebocytes to the sites of infection [141]; the vibrio apparently bypasses these defense reactions. This mass mortality of corals was recently matched by *V. cholerae* in human mortality in Zimbabwe [142].

Zooxanthellae-carrier and heat-shocked corals release diverse virus-like particles of varying morphologies: tail-less hexagonal particles of 40–50 nm diameters, droplet-shaped particles, and filamentous particles, all unidentified. It was not determined if these viral particles were of coral- or alga-derivation [143]. Ultraviolet-irradiated symbiodinium cells released filamentous virus particles resembling *Closteroviridae* RNA plant viruses [144]. In terrestrial plants (like citrus trees in Florida), these ssRNA tristeza citrus viruses are transmitted by toxoptera aphids [145]. The integrated and activated genomes of the algal filamentous viruses are similar to lysogenic bacteriophages, thus these hosts are subjected to genes horizontally inserted.

Diatoms and dinoflagellates maintain ancient host-virus relationships. The photosynthetic diatoms (*Bacillariophyceae*), contributors to the Earth's oxygen level, foodstuffs for zooplanktons and for various larvae, are harboring tail-less icosahedral ssRNA viruses. The RsRNAV (*Rhisosolenia setigera*) icosahedral ssRNA virus and the Cten virus (*Chaetoceros tenuissimus*) infect diatoms. The Cten virus in its lytic cycle, appears as a very large progeny of new viral particles released: 10^{10} infectious units/ml. These viruses form a new family, BacillarioRNAviridae/*Bacillariophyceae*. In contrast, the CsNIV (*C. salsugineum* nuclear inclusion virus) has a ssDNA genome [146, 147], thus representing a different, but non-interfering class if viruses. Viral interference is often replaced by viral coexistence.

The blooming and toxic photosynthetic dinoflagellate member of *Dinophyceae*, the *Heterocapsa circularisquama*, harbors two ancient viruses: a positive sense ssRNA virus and a dsDNA virus. In this host, the RNA and the DNA viruses tolerate each other. In the human host, DNA herpes- and RNA retroviruses synergize with one another (*vide infra*). The HcRNAV encounters sensitive, resistant and delayed lysis-udergoing hosts. The initiation codon for viral replication is the universal AUG. Sensitive hosts undergo rapid lysis and release viral particles. Resistant cells inhibit viral replication. Viral ORF-1 encodes the RdRp (RNA-dependent RNA-polymerase). The phylogenetic derivation of some land viruses (mushroom bacilliform virus, and others) from marine viruses is supported by the similarities of their RdRp to that of HcRNAV. Viral ORF-2 encodes the viral coat protein [148–150].

A giant dsDNA virus, HcDNAV, infects and lyses *H. circularisquama* cells and thus it can control this blooming toxic dinoflagellate. The viral capsid is icosahedral up to 210 nm in diameter; the DNA genome consists of 356 kbp. Viral replication takes place in the cytoplasmic viroplasms. By its looks, the virus was made a member of the PhycoDNAviridae, but by its biochemistry it does not fit into that group of large marine dsDNA viruses (*vide infra*). The amino acid sequence of its PolB (type B DNA polymerase) gene differed from that of the phycodnaviruses. This gene sequence was related closest to that of the Asfarviridae member, African swine fever virus (ASFV). Thus, this ancient marine virus and the much later evolved terrestrial mammalian virus have preserved their phylogenetical relationship [151].

Algae are generous gene-donors: stress-related algal genes (ascorbate peroxidases, metacaspases) operate in choanoflagellata [152]. The green alga *Paramecium bursaria* chlorella virus, and the coccolithovirus of *Emiliania huxleyi* (EhV) fit into the group of PhycoDNAviridae, the nucleocytoplasmic large dsDNA viruses. The haptophyta *E. huxleyi* represents one of the deepest branching lineages of the eukaryotic tree of life. Its exoskeleton displays carbonate scales, the coccoliths. The icosahedral 1,900 Å diameter chlorella virus (PBCV-1) genome encodes 365 proteins and is capable of glycosylating its major capsid proteins (without any help from the host cell's endoplasmic reticulum and Golgi apparatus). Beneath the glycoprotein capsid, lipid bilayer membrane surrounds the dsDNA core [153, 154]. The coccolithovirus EhV enters its host cell of the diploid calcified phytoplankton microalga *E. huxleyi* by envelope fusion and endocytosis [155]. The haploid phase of the *E. huxleyi* life cycle disallows viral entry and survives uninfected [156]. EhV encodes cytotoxic glycosphingolipids, which induce apoptotic death in infected cells [157]. There are seven sphingolipid biosynthetic genes (named in the article), one of them is longevity assurance factor, LAG1: wise little creature, *E. huxleyi*! EhV acquired these host genes *via* horizontal transfer; or *vice versa*, was it the virus that transferred these genes to its host? The virus needs lipid metabolism to construct its lipid membrane. Since lipid metabolism was present in eukaryotic cells ancestral to *E. huxleyi*, the

direction of gene transfer was very likely from host cell to its virus [158]. The picoeukaryotic photosynthetic green alga, *Ostreococcus tauri* harbors a large linear dsDNA virus, OtV5, a phycodnavirus. The host cell is so small, and a virus particle is so large, that at burst only 25 virus particles can be released. One of the viral coding sequences (CDS, flanked by start and stop codons) encodes praline dehydrogenase. This stress-reactive enzyme is coming from a horizontally transferred gene, probably bacterial-to-algal-to-viral derivation [159].

While the algal viruses may pick up host cell genes, being eukaryotic viruses, they could not integrate their genomes into bacteria; thus, these algal viruses can not be depicted as vectors of the dinoflagellate toxin genes to higher mammalian hosts, which they may not even be able to infect. The *Asfarviridae* hemorrhagic swine fever virus (ASFV) acquired its PolB gene on a long distance phylogenetic scale (*vide supra*). However, dinoflagellates may associate with bacteria (among them phage-carrier vibrios), which may acquire dinoflagellate toxin genes by horizontal transfers. These toxin genes may flank of, or fuse with, integrated phage genomes and thus may be spread by promiscuous phages to new bacterial hosts pathogenic to mammalian, including human, hosts.

2.2.2 Genes of the Mimivirus Shared with Its Phage and with Its Host Amoeba

The large nuclear-cytoplasmic dsDNA viruses (poxviruses, asfarviruses, iridoviruses, phycodnaviruses) show monophyletic origin. Poxviruses are grouped with asfarviruses and mimiviruses are grouped with iridoviruses and phycodnaviruses. In a most peculiar way, divergence of these viral families appears to have predated the divergence of the major eukaryotic lineages. After the divergence of the host cell lineages, further evolution of the viral genomes occurred due to horizontal receipt and incorporation of genes from their host cells and co-infecting bacteria and viruses. These large dsDNA viruses co-evolving not only with dinoflagellates, but with animal hosts (asfarviruses, iridoviruses, mimiviruses, mimicking microbes) acquired anti-apoptotic and immunosuppressive genes. The genes acquired from bacteria include bacteriophage genes [160, 161]. The viral B family DNA polymerases (PolB) are conserved in the large dsDNA viruses, herpes- and baculoviruses and show low frequency of recent horizontal transfers. Comparable PolB genes occur in archaea viruses and in the three archaeal lineages (*Nanoarchaeota, Crenarchaeota, Euryarchaeota*). In the mimiviridae group, the 16 PolB fragments underwent substantial segment variations. Viral PolBs show much higher diversity than bacterial PolBs [161].

Acanthamoeba polyphaga harbors mimivirus; with its 750 nm size and 1.2 Mbp genome, it is the largest known dsDNA virus. Mimivirus is closely related to the large dsDNA algal viruses. The ancestral mimivirus infected microalgae of the phytoplankton. The mimiviral genome containing both DNA and RNA stands on the boundary between viruses and bacteria (or even eukaryotic protocells). Of the four mimivirus aminoacyl-tRNA synthetase genes, two (TyrRS; MetRS) are archaea/eukaryota-related, except for their anticodon binding sites [162]. Mimivirus-like PolB and amino acid sequences occur in algal phycodnaviruses of the Norwegian coastal waters: CeV01 (*Chrisochromulina ericina*), PpVo1 (*Phaeocystis pouchetii*) and PoV01 (*Pyramimonas orientalis*). The mimivirus PolB sequence exhibits 45, 41, and 31% identity with the PolB sequences of PoV01, CeV01 and PpV01. These icosahedral viruses are smaller than the mimivirus, being of 160–220 nm in diameter with genome sizes of 485–560 kb. For comparison, the genome of the phycodnavirus EhV-86 is 407 kb [155, 163]. In the host amoeba, the mimivirus genome is allowed to grow rather than retract. Instead of genome reduction it gains genome size by the acquisition of horizontally transferred genes and mobilomes. The viral genome enlarges within the amoeba by lineage-specific gene duplications, lateral gene transfers from the host and accretion of mobile genetic elements (transposons and retrotransposons) [164]. It was proposed that poxviruses accept host cell mRNAs reversely transcribed into cDNA, ready for integration. Among horizontally acquired poxvirus genes are those of IL-10, thymidine kinase,

ribonucleotide reductase, glutathione peroxidase (for oxidative damage protection), deoxyribopyrimidine photolysase (for repair of ultraviolet light damage). The IL-10 gene in the poxvirus genome (yatapox, canarypox viruses) is the result of horizontal transfers from eukaryotic hosts [165]. Ancient poxviruses vectored retrotransposons from reptiles to mammals [166]. The amoeba is a "melting pot of genes and evolution" in reference to mimivirus and the 368 kb genomic Marseillevirus residing in it [167, 168].

The arrangement of major capsid proteins of large dsDNA viruses (chlorella virus, PBCV1; *Sulfolobus* turreted icosahedral virus, isolated from the acidic hot springs (90°C) of Yellowstone park, where it infects the hyperthermophilic crenarchaea *Sulfolobus solfataricus* (*vide supra*), the photosynthetic marine cyanobacteriophage PM2, and coliphage Period A, PRD1, show by X-ray crystallography close identity. Even though these viruses infect host cells of wide variety (archaea, prokaryota, eukaryota), they derive from a common precursor [169, 170]. The crenarchaeota viruses, however, display unique features suggesting an evolutionary path with gene gains from both their hosts and also from prokaryota [171]. The enveloped dsDNA acidianus filamentous virus-1 (AFV-1) of the *Lipothrixviridae* class infects a crenarchaeal host in the acidic hot springs of Yellowstone Park and its gene repertoire is quite unique [172].

The organization of the mimivirus capsid, the starfish-shaped arrangement of its capsomers and fiber components, indicates that its encoding genes are of prokaryotic and eukaryotic derivation [173]. Viruses (coronavirus; human immunodeficiency virus-1 (HIV-1)) usually utilize host cell cyclophilins; mimivirus encodes its own cyclophilin [174]. The mimivirus genome can encode 911 proteins; of these, 298 have recognized functions (translation enzymes, DNA repair pathways, topoisomerases). This virus resides also in corals and sponges [175, 176]. The two cytochrome p450 genes of the mimivirus show 23–26% identity with bacterial (*Proteus mirabilis*), fungal (*Aspergillus*), streptomyces (*S. peucetius*) and caenorhabditis cytochromes. The short region of mimivirus dysferlin (Ca^{2+}-binding protein) has 35% homology with that of the sea urchin (*Stongylocentrotus purpuratus*). The ADP-ribosyltransferase pierisin-1 shows 24% identity with that of *Pieris rapae* (the lepidopteran pest of cruciferous vegetable crops) [177, 178].

If large dsDNA viruses existed before cells were formed, then viral genes were donated to protocells, and not *vice-versa*. The transcriptional gene silencing Tgs eukaryal enzymes methylate the N2 atom of-7-CH_3 guanosine nucleotides. The *Giardia* Tgs protein and the mimivirus Tgs protein are unique and similar in their capacity to methylate guanine-N2 in the absence of prior N7 methylation [179].

In addition to amoebae, corals and sponges may host as yet unidentified mimivirus relatives [175, 176]. Of the new proteins encoded by the mimivirus genome there are four aminoacyl-tRNA synthetases. Several non-coding RNAs are prominently expressed. Gene expression late promoters of the mimivirus were shared with its phage, the sputnik. Polyadenylated transcripts derived from new gene (previously unknown: no database homolog) with gene product proteins of unknown function. Mimiviral tRNA methyltransferases and aminoacyl-tRNA synthetases (new for a virus to encode it) are activated early. In response, a burst in the transcription of mitochondrial genes of the amoeba takes place. This event coincides with the viral "eclipse phase", during which neither formed, nor functional viral elements are present in the viroplasms. The eclipse phase is followed by the upsurge of mimiviral, and the decrease of amoebal gene transcripts. For comparison, Fig. 2.1 shows the eclipse phase of influenza A virus adapted to chicken embryo, or to mouse lung (Fig. 2.1) [180]. The synthesis of the LPS-like outer layer of the viral particle is a late event. Mimiviral genes encoding capsid proteins and collagen-domain proteins are among the last to be activated [181]. The cellular nucleoside diphosphate kinases are small 150 aa proteins highly conserved within archaea, bacteria and eukaryota (>40% identity). The cellular nucleoside diphosphate kinases (NDKs) do not distinguish ribonucleotides from deoxyribonucleotides, acting equally in both, transferring phosphate groups from nucleoside triphosphates (NTP, other than adenosine triphosphates, ATP) to nucleoside diphosphates (NTP to NDP). In contrast to the cellular enzymes, mimiviral NDKs display distinct affinity for deoxypyrimidine

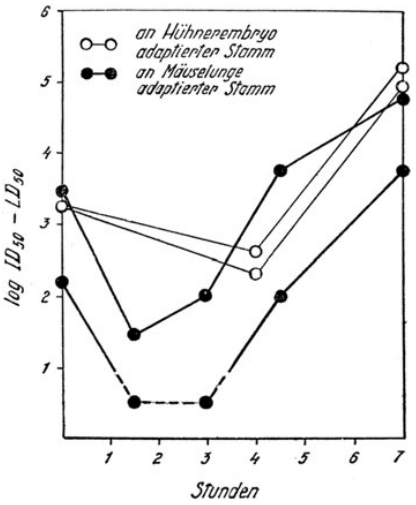

Fig. 2.1 Graph from "Die Grundlagen der Virusforschung (1956)" showing the disappearance ("eclipse") of all detectable viral activity ("die Dauer des Vermehrungszyklus die Infektionsfähigkeit verliert") after the inoculation of influenza A virus into the allantois cavity of a chicken embryo, or into a mouse lung, and the reappearance of viral structural proteins and then mature extracellular infectious virions. The work started in 1950 and was published by Sinkovics and Molnár in 1954 [818, cited in 180]. Permission to re-publish is from Akadémiai Kiadó, Budapest

nucleotides. The mimiviral enzyme is ancestral to the cellular (including that of the amoeba) NDK enzymes. The mimiviral enzyme clusters with euryarchaeal, crenarchaeal and bacterial sequences. The Acantamoeba (*A. castellani*) enzyme clusters with fungal, and metazoan sequences. The viral enzyme was not acquired from a eukaryotic organism by horizontal gene transfer [182].

An icosahedral dsDNA virus 50 nm in size packing a 18 kb circular genome replicates only in the viroplasm of the mimivirus. In the presence of the replicating "sputnik", the mimivirus can not mature into complete particles; its capsid assembly becomes distorted. It is not lysed; yet the sputnik acts like a phage to the mimivirus: a "virophage". Three sputnik genes encode proteins of mimivirus derivation; other sputnik genes encoding integrase, helicase, ATPase and transposase are of bacteriophage and eukaryotic viral derivations. The sputnik displays a double jelly-roll capsid. Sputnik lacks RNA- and DNA polymerases and borrows mimiviral transciptosome. The palindromic signal characteristics of mRNA polyadenylation sites in many sputnik genes are those of the mimivirus; so is its late protein element [181, 183, 184].

Intraamoebal bacteria, *Legionella drancourtii* and *Coxiella burnetii*, possess a gene of aquatic viridiplantae origin. This gene encodes the enzyme sterol delta-7 reductase; the intraamoebal mimivirus also transcribes this gene. The host amoeba generously donates its genes to its parasites, the intraamoebal viruses and bacteria. The intraamoebal parasites exhibit genomes larger than that of their extraamoebal relatives. However, in the case of the sterol delta-7 reductase and another eukaryotic enzyme, the ATP/ADP translocase, the gene donor was not the amoeba, but a chlamydia. The chlamydia acquired the gene from a parasitized aquatic plant. Upon transferring itself into the amoeba, there the chlamydia encountered other guests of the amoeba. Through another horizontal transfer, these genes were implanted from the chlamydia to the bacteria (and to the mimivirus) [185].

The *Acanthameba polyphaga mimivirus* (APMV) has the potential to infect the lungs of intubated patients in the intensive care units. Macrophages of the respiratory tract take up the mimivirus by phagocytosis. Patients with ventilator-associated pneumonia develop antibodies to mimivirales [186–188].

2.3 Interviral (Virus-to-Virus) Gene Transfers

2.3.1 Marek's Disease Herpesvirus

"Multiple Nervenentzündung (Polyneuritis) bei Hühnern" described in 1907 in the *Deutsche tierärztliche Wochenschrift* by the Hungarian veterinarian József Marek was a new disease entity characterized by heavy lymphocytic infiltrates in multiple organs, but especially in nerve sheaths and in the meninges. The causative agent of the "Mareksche Geflügellähmung", which was recognized to be a neoplastic entity, was transferable by filtrates. Thus, the viral etiology of the lymphomatous tumors was declared [189]. The replication of the Marek virus in tissue cultures was shown first in B. R. Burmesters's laboratory [190, 191]. The first Marek's herpesvirus strains were isolated in chicken kidney cell cultures by J. L. Spencer [192]. The co-operative interactions in malignant lymphoma induction between Marek's herpesvirus and chicken leukosis and chicken sarcoma (Rous) retroviruses were first documented in tissue cultures at Rutgers, the State University of New Jersey, by J. W. Frankel and Vincent Groupé [193] and confirmed and elaborated on at Columbia University in New York and in the germ-free animal laboratories of Life Sciences in St. Petersburg, Florida [194].

2.3.2 Reticuloendotheliosis Virus Genomic Sequences in the Marek's Virus and in the Fowlpox Virus Genomes

Leuko-, lympho- and sarcomagenic retroviruses were recently shown in a condensed tabulated form [195]. The T strain of chicken reticuloendotheliosis virus (REV-T; REL) transforms chicken lymphocytes, but not chicken embryonic fibroblasts. This virus infects both CD4 and CD8 T lymphocytes [196]. The length of its sequenced proviral genome is in the range of 8,284 nucleotides [197]. The *v-rel* oncogene and its cellular homologue *c-rel* encode the p59v-Rel phospho-oncoprotein. The oncoprotein remains in the cytoplasm of transformed spleen cells, but in transformed E26 myeloid cells the p59v-Rel oncoprotein translocates from cytoplasm into the nucleus [198]. The natural protein Rel/NF-κB is an inducer of inflammatory and immune responses, promotes cell-proliferation and it exerts anti-apoptotic effects. The viral oncoprotein transforms lymphoid cells and induces malignant tumors resembling human mediastinal B cell lymphomas and Hodgkin's disease. In the human counterparts of such tumors, overexpressed or mutated c-Rel oncoprotein activates the expression of anti-apoptotic and pro-proliferative genes in the nucleus. The vRel oncoprotein promotes the expression of telomerase reverse transcriptase (TERT), thus preventing the shortening of telomeres at cell divisions.

The viral transcription activation domain (vTAD) interacts with CAPERα, which synergistically modulates the transactivation by vTAD. The co-activator of activating protein-1 (AP-1) and estrogen receptors (CAPERα) is expressed in liver cirrhosis and in hepatocellular carcinoma. In vRel-mediated lymphomagenesis, CAPERα acts as a transcriptional co-regulator and antagonist of the transforming activity of vRel oncoprotein. Neutralizing CAPERα mRNA by siRNA in vRel-transformed lymphoma cells increased the cells malignancy, as expressed by their enhanced colony formation. Tumor suppressor CAPERα has to be silenced by siRNA or by disabling mutation for the cRel oncoprotein to act uninhibited in human lymphoma cells. In human Reed-Sternberg cells of Hodgkin's disease, cRel is an active oncogene. In avian malignant lymphomas transcriptional co-activation of vTAD by CAPERα is promotional to its antagonism for vRel's transforming activity [199]. Three oncogenic viruses, avian leukosis virus (ALV), reticuloendotheliosis virus (REV) and Marek's disease virus (MDV) down-regulate the expression of the non-coding tumor-suppressive gga-microRNA-26a (*Gallus gallus*), the regulator of tumor suppressor PTEN gene (phosphatase and tensin homologue deleted on chromosome ten). One of the tumor-suppressive effects of gga-miRNA-26a is antagonism to the "T cell growth factor" IL-2 expression in these avian tumors [200].

The JARID2 gene product histone lysine demethylizing proteins (Jmj, jumonji, cruciform in Japanese) bind to cyclin D1 promoter and repress the transcription of cyclin D1. Jmj forms complexes with histone methyltransferases and reacts with the cyclin D1 promoter, thus increasing histone methylation, while cyclin D1 is repressed. Jmj family proteins regulate both methylation and demethylation of histones (J. jumonji in Japan; ARID, AT-rich interaction domain) [201]. In chicken B cell lymphomas, REV-T induces the oncogenic miRNA-155 for targeting JARID2. Part of the histone methyltransferase complex, JARID2, rapidly loses its pro-apoptotic activity under the effect of the antagonistic miRNA-155. The pro-survival function of miRNA-155 promotes lymphoma cell growth in the absence of JARID2 activity [202].

Phylogenetic relationship of gallid herpesvirus-2, MDV, did not reveal how its oncogenes *meq* and *pp38* evolved [203]. The MDV encodes oncoprotein Meq, a homologue of cellular proto-oncogenes *fos* and *jun* (*fos*, Finkel osteosarcoma murine retrovirus oncogene; *jun*, ju-nana Japanese for seventeen, after avian sarcoma virus ASV-17). MDV also encodes the immediate-early transactivator protein, ICP4, to which a small antisense RNA is expressed in lymphoma cells [204]. MDV-related abbreviations: the lytic antigen pp38 (Meq, Marek's EcoRI-Q DNA restriction one minifragment probe, Eco from *Escherichia coli*; pp38, phosphoprotein-38). The MDV-encoded RNA telomerase subunit (vTR) shows 88% sequence identity with the chicken gene cTR. The MDV vTR maintains telomere length in transformed cells [205]. The virus releases a number of miRNAs (similarly to Epstein-Barr virus, EBV, and HHV-8, Kaposi sarcoma-associated herpesvirus, KSHV). The MDV miRNAs interact with viral oncogene *meq* and with the viral latency associated transcript (LAT) [206]. MDV may either lay latent in avian CD4 T cells, or it malignantly transforms them. In a REV-transformed lymphoid cell line, latent MDV expressed its Meq oncoprotein antigen and contributed to the cells' apoptosis resistance. Treatment with bromodeoxyuridine induced the expression of MDV lytic antigens [207]. The Meq oncoprotein due to its Pro-Leu-Asp-Leu-Ser motif binds C-terminal binding protein (CtBP), a transcriptional co-repressor. The Meq-CtBP complex is essential for oncogenesis: cells with mutated CtBP are exempted from MEq-induced oncogenesis. The EBV oncoprotein EBNA3A/3C (nuclear antigen) also interact with CtBP. MDV vaccine strains do not encode the Meq oncoprotein and do not interact with CtBP [208].

Syngeneic and allogeneic cell-mediated cytotoxicity against Marek's disease virus- (MDV-) transformed lymphoblastoid tumor cell lines revealed brisk alloantigen-directed (allogeneic) reactions, and rare syngeneic reactions by host lymphocytes [209]. Targeting lymphocytes against specific oncoproteins (phosphoprotein pp38 of MDV), or harvesting them from REV-sensitized donors, yielded virus-specific syngeneic cytotoxic lymphocytes [210]. Splenic lymphocytes of MDV-sensitized donors killed transformed syngeneic lymphocytes expressing MDV oncoprotein antigens pp38 and Meq [211].

These types of lymphocyte-mediated immune reactions directed at human sarcoma cells were observed already in the late 1960s and early 1970s in the author's laboratory at M. D. Anderson Hospital, Houston, TX. The autologous (or syngeneic) reactions were mediated by immune T cells, whereas the allogeneic reactions were mediated by large granular lymphocytes that were later designated to be natural killer (NK) cells [212–214]. These observations are documented and illustrated in Sinkovics' monograph "Cytolytic Immune Lymphocytes. . ." (Fig. 2.2a,b) [213].

REV-transformed avian T-lymphoblastoid cells accept co-infection with MDV [215, 216]. The two avian T cell lymphoma viruses (MDV and REV) synergized their pathogenicity, as the MDV genome accepted the insertion of the REV genome. Tumor cells co-infected with these two viruses either in vitro or in vivo, yielded MDV isolates that accepted the insertions of retroviral genomic segments in their genomes. It was the long terminal repeat (LTR) of REV that penetrated the MDV genome; both attenuated and virulent strains of MDV exhibited such insertions [217]. Herpes- and retroviruses were known to synergize their infectious and cell-transforming processes by augmenting each other's efficacy in malignant transformation [193, 218]. The Rous sarcoma virus LTR was transactivated by MDV [219]. The LTR of HIV-1 yielded to transactivation by human herpesvirus-1 (HHV-1) and other

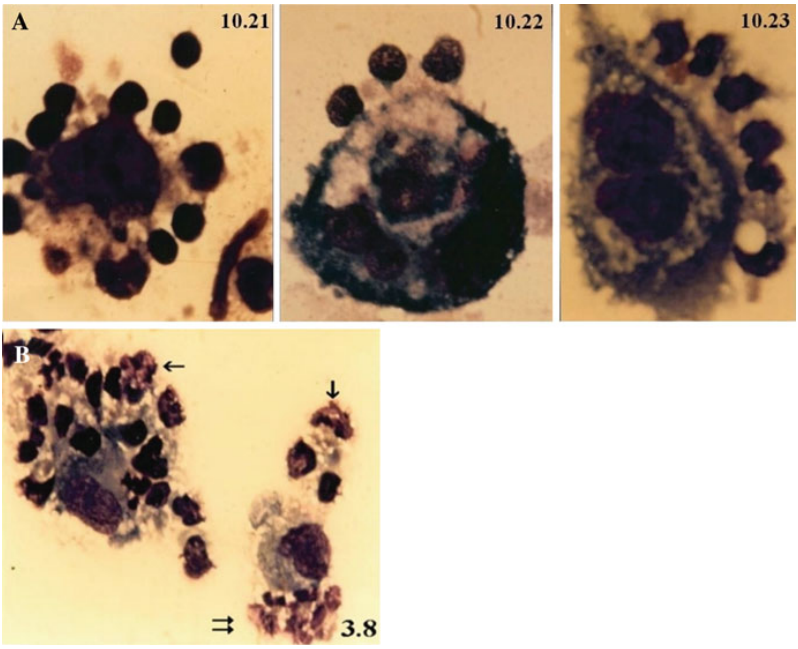

Fig. 2.2 (a) Human sarcoma cells attacked and lysed by autologous lymphocytes. The compact small round cells are immune T cells. It appears as if the lymphocytes injected "cytolysins" into the cytoplasms. The large granular lymphoid cells in 10.21 are NK cells. (**b**) In 3.8, human sarcoma cells withstand attack by a mixed population of lymphocytes; some lymphocytes die apoptotic death (*arrows*) next to the attacked tumor cell. From the Section of Clinical Tumor Virology & Immunology, M. D. Anderson Hospital, Houston, TX, in the early 1970s [213]. Permission to re-publish is from Schenk Buchverlag, Passau and Budapest

DNA viruses [220]. *Vice-versa*, MDV transactivated the promoters of avian leukemia and sarcoma (Rous) viruses [221, 222].

The phenomena of retroviral genomic segmental insertions into the genome of DNA viruses were observed naturally and induced artificially. The two avian T cell lymphoma viruses, MDV and REV, synergized their pathogenicity as the MDV genome accepts inserted segments of the REV genome [217] Transcripts of the REV's LTR promoters enhanced the expression of MDV US (unique short) genes [223, 224]. Insertions of the retroviral genome occur within one or two passages in MDV-infected cells. The retroviral insertion sites are two, 1 kb region each at the junction of the short unique and short repeat regions of the MDV genome. To the malignancy of a MDV-transformed lymphoma cell line, REV contributed the activation of the *c-myb* (myeloblast) proto-oncogene [225]. A clone of MDV derived through REV genomic insertion exhibited attenuated oncogenicity, but remained infectious by contact, caused thymic and bursal atrophy and induced severe immunosuppression [226]. In MDV- and REV-co-infected cells, chimeric molecules were formed from REV-LTR and MDV flanking proteins [227]. In comparing the horizontal transmissibility of MDV-GX-0101 field strain harboring the LTR of REV, LTR-deleted viral clones were more immunosuppressive and less transmissible through horizontal routes, than the original REV LTR-positive strain [228].

Fowlpox virus (*Avipoxvirus*) vaccines were frequently contaminated with REV. Fowlpox vaccine virus isolates in the chorioallanois membranes of chicken embryos grew in lesions yielding both fowlpox virus and REV. It could not be distinguished if proviral REV DNA was integrated in the

cells' DNA and/or into fowlpox virus DNA. The REV-contaminated fowlpox virus vaccine in inoculated chickens caused fowlpox lesions and feathering defects and proventriculitis [229]. In fowlpox virus field isolates, the integrated genomic segments of REV *env* gene were identified. Fowlpox virus vaccines carrying integrated REV genomic sequences induce weak protection against fowlpox [230]. In some fowlpox vaccine viruses, the full genome of REV was found integrated. These integrated REV full length genomic sequences (*gag, pol, env* and LTR) are replication competent [231], *gag,* group-associated antigens are the virions' structural proteins, *pol*, polymerase, encodes the viral enzymes: protease, integrase and reverse transcriptase; *env*, envelope proteins. REV-free field isolates of fowlpox virus (both chicken and turkey) exist. Some fowlpox virus isolates from wild birds or from poultry harbor none, or only remnants of REV LTR, and no REV *env* genes [232] The production of REV-free fowlpox virus vaccines is possible. A refined quantitative multiplex real time polymerase chain reaction (PCR) is available to select out REV-free fowlpox virus strains [233]. It is with the MDV vaccines and the fowlpox virus vaccines, where the biological importance of horizontal viral gene transfers and recombinations between unrelated viruses are surpassed by the practical urgency of the problem.

2.3.3 Herpesviruses Activate Latent Retroviruses

Over ten million years of coexistence in sharing the same hosts in Africa and co-evolving with each other and with their simian and hominid hosts, created a special relationship between herpes- and retroviruses. Instead of interference with each other, herpes- and retroviruses co-operate. Their cooperation consists of either suppression of lethal infections by the partner virus in the interest of keeping the host alive; or of the activation of the other virus to gain growth factors (cytokines and chemokines), have access to transformed host cells protected against apoptotic deaths, into which to integrate, and benefit from immunosuppression of the host by creating a Th2-type environment, and thus eliminating IFN-γ and TNF-α production in the host. From the human point of view, the cooperation between herpes- and retroviruses is a form of "criminal collusion" [213]. The MDV increases the rate of transcription of the avian leukemia virus, Rous-sarcoma-associated retrovirus (RAV-2 ALV). Five- to tenfold RAV-2 AVL RNA and viral structural proteins are produced in the presence of MDV [221]. The phenomena of latent retrovirus activation in herpesvirally co-infected human tumor cells have been well recognized [234–241]. The widely spread leukemogenic-sarcomagenic retrovirales of vertebrates from the fish up to Old World simians are not expressed in human leukemia and sarcoma cells [195, 213]. However, in herpesvirally (HHV-8; KSHV) induced human Kaposi sarcoma cells there appears an activated endogenous retrovirus (Fig. 2.3a,b) [213]. If there is a latent human sarcoma retrovirus not lost during human evolution and still is in hidden existence, it may be found in EBV-infected leiomyosarcoma cells of children [213].

2.3.4 Avian Herpesviruses Descend from Theropod Dinosaurs?

The ancestors of alligators, crocodiles and turtles coexisted with dinosaurs (*deinos,* Greek, terrible; *sauros*, Greek, lizard) and avian genera descended from feathered "bird-footed" "beast-footed" theropod (*therio,* Greek, wild carnivorous beast) dinosaurs. The ancestors and the "missing links" are preserved in the Gobi desert and elsewhere (Liaoning, China) possessing melanosomes for the spectacular coloring of their skins and plumage [242–247].

There is a gap between extant herpes- and retroviruses of crocodilians, reptilians, amphibians and *Aves* represented by the viral flora of the extinct dinosaurs (*Archosauria)*. Turtles are at the base

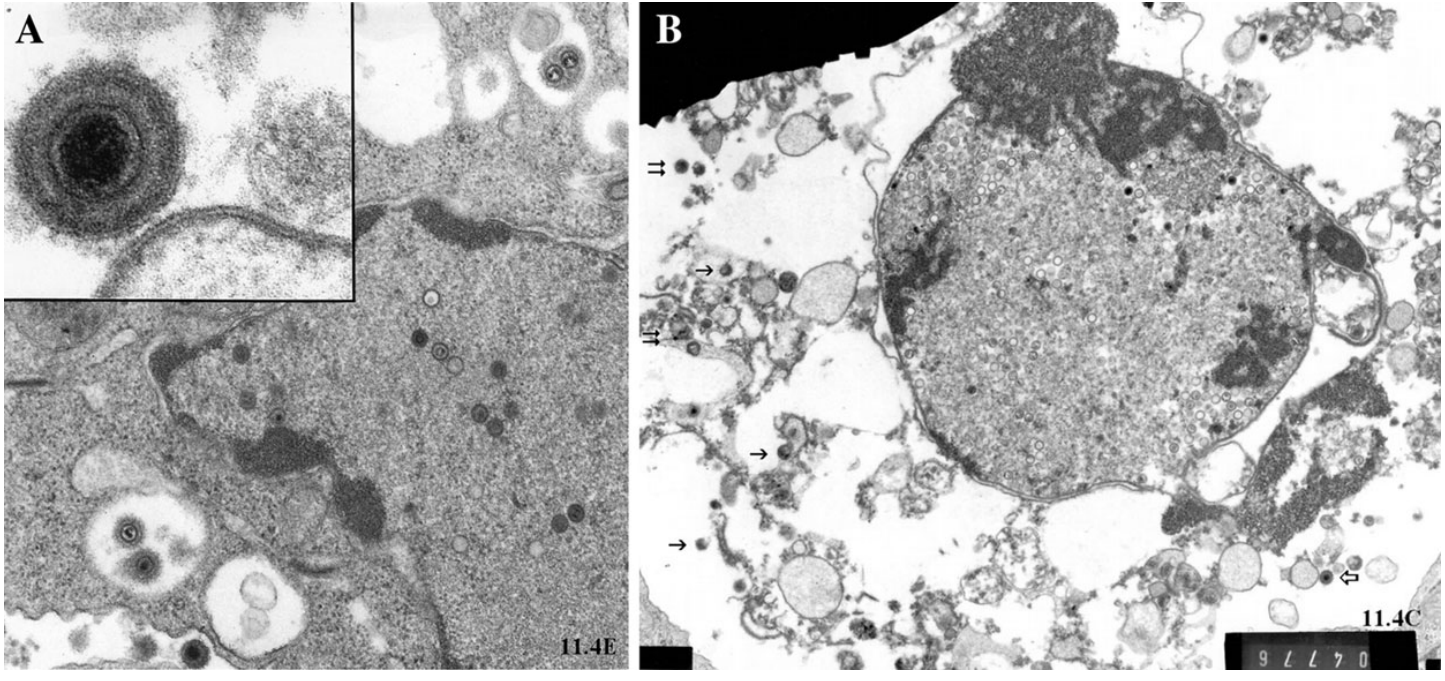

Fig. 2.3 (a) Classical Mediterranean Kaposi's sarcoma cells from the pre-AIDS era, in the early 1970s. Herpesvirus particles are those of HHV-8 (KSHV) (not known at that time). (**b**) Unidentified budding retrovirus particles (different in morphology from HIV-1) are those of an activated endogenous retrovirus (*four arrows*) in the disintegrating cytoplasm of a Kaposi's sarcoma cell. Next to the nucleus (*single arrow*) a mature herpesvirus-like particle is present (HHV-8, unidentified). The cell nucleus contains immature herpes-like virus particles. From the Department of Pathology (chief, Prof. Ferenc Györkey†), Veterans' Administration Hospital Medical Center, Houston, TX [213]. Permission to re-publish is from Schenk Buchverlag, Passau and Budapest

of the crocodile-bird branch [248, 249]. Reptilian α-herpesviruses cause the chelonid fibropapillomatosis in marine turtles (*Chelonia* sp.). Marine leeches (*Ozobranchus* sp.) vector huge loads of the fibropapilloma-associated turtle herpesvirus [250–256]. The herpesviruses infecting loggerhead turtles (*Caretta caretta*) cause fibropapillomatosis, tissue ulceration and necrosis and cell syncytia formation with intranuclear inclusion bodies [257]. Thus, new families of Herpesviridae emerge to include the bivalve (oysters), fish, reptile, turtle and tortoise, lizard, snake, crocodilian and amphibian herpesviruses [258, 259]. The herpesviruses form three distinct groups: (1) reptilian, avian and mammalian (reflecting to "ancient coevolution of these virus lines with the development of birds and mammals from reptilian progenitors"); (2) fish and amphibian; and (3) invertebrate bivalvian herpesviruses [260, 261].

Tortoises (*Testudinidae*) carry pathogenic herpesviruses causing glossitis and gastritis. Multifocal hyperemic-hemorrhagic nodules and plaques of the cloacal and phallic mucosa of juvenile alligators (*A, mississippiensis*) yielded isolates of tortoise herpesvirus-1; the lesions were infiltrated by monomorphic round cells resembling monoclonal lymphocyte populations. The isolates fitted into a phylogenetic tree of α-herpesviruses in comparison with turtle, tortoise, crocodylid and varanid herpesviruses; other herpesviruses in this phylogenetic tree were the gallid HV-2 (Marek's virus), bovine, equine, feline and human herpes simplex viruses-1, 2 and psittacid HV-1 [262]. The psittacid HV-1 causes cloacal papillomatosis in the Amazonian parrots (*A. aestiva*). These birds eventually succumbed to bile duct and pancreatic adenocarcinomas. The principle of Koch's postulates for herpesviral etiology for the adenocarcinomas has not been satisfied [263].

In the evolution of *Aves* in the families of *Archosauria*, there were gene preservations and gene losses. Mitochondrial DNA sequences identify *Archosauria* descendants (crocodilians and birds) and *Lepidosauria* (lizards and snakes) [264]. Conserved nuclear genomic sequences are reptilian olfactory receptor genes in birds [265]; melanosomal matrix genes [266]; the interspersed repetitive elements of chicken repeats (CR1) [267]; alligator isochors as GC-rich bird isochors [268]; the glycine-proline-tyrosine rich beta-keratin protein similarities in crocodilians and birds; reptilian beta-keratins as glycine-rich feather keratins and cornifications in feather morphogenesis [269–271]. Tuatara (*Sphenodon* sp.) reptilian chromosomal segmental regions show homology and orthology with chicken chromosomal segments, among them some tuatara autosomal regions are homologous with the sex chromosomes of birds and mammals [272].

Sex chromosomes remain highly conserved: chicken Z chromosome corresponds to turtle chromosome 6q, snake chromosome 2p and crocodile chromosome 3 with the order of genes preserved. The absence of homology between bird Z chromosome and snake and turtle Z sex chromosome indicates that these sex chromosome genes have had different origins [273]. Nucleotide and aa sequence alignment of saltwater crocodiles' oocyte maturation factor (C-mos) display strong similarities with that of birds (chicken and zebra finch) [274]. Ancient syntenies are conserved in fish, reptilian, avian and mammalian microchromosomes [275]. The chemokine IL-8 (CXCL8) shows up first in the reptilian turtle genome [276] and remains conserved through birds [277–279] and mammals up to *Homo* [280].

Genomic sequences "lost in translation" are IgD of the gecko missing in birds [281]; the Na^{+}-Ca^{2+} exchanger NCX family regulators of teleosts, amphibians and reptilians missing in birds and mammals [282]; of the tooth-forming genes, the dentin matrix protein-1 gene lost in birds [283].

The abundant presence of retroviral sequences in vertebrate genomes so far failed to detect the evolutionary connections between reptilian progenitors of birds and mammals [284]. In extant descendants of *Archosauria*, the crocodiles and birds, the CR1-like retrotransposons are active and the encoded C-mos (*vide supra*) between crocodiles and birds reveal significant sequence similarity [274]. Retrovirally (avian sarcoma retrovirus) mediated gene insertions into young chickens revealed those genes that are essential to feather morphogenesis. These genes are those of the bone morphogenetic protein-4 (BMP) for interacting with noggin (BMP antagonists noggin and dickkopf) to induce rachis formation, barb fusion and barb branching; and for the sonic hedgehog protein (Shh) to remove by apoptosis induction marginal plate epithelial cells in between barbs [285]. Was it retrotransposons that

horizontally inserted such genes into theropod dinosaurs? Were the first flights of the pterosaurs 250 million years ago bat-like or fathered bird-like? Pterosaurs were flying 150 million years before bats, and 70 million years before birds, whereas the first placental mammals appeared just over 100 million years ago. In the Cretaceous-Tertiary boundary 65 million years ago, the first primates diverged from ungulates and a cataclysmic extinction of the dinosaurs occurred, but the crocodilian ancestors survived [286]. The *Pan* and *Homo* lineages separated about 7 million years ago, and *Australopithecus afarensis* lived 3.7 million years ago in what is Hadar, Ethiopia, today. Both birds and flying bats constricted the sizes of their genomes; retrospectively calculated (Markov chain Monte Carlo approach), pterosaurs operated with a much constricted genome [287]. Conserved genomic segmental overlaps occur between alligators, turtles, emu and chicken, despite a drastic reduction of the chickens genome size in comparison with the sizes of the reptilian genomes [288]. Even though a scrutiny of herpes- and retroviral agents active in birds, especially the Marek's virus (*vide supra*), so far failed to identify with exact precision the reptilian or amphibian ancestors of these avian viruses (*vide infra*), the descent of *Aves* from theropod dinosaurs is strongly supported by genetic evidence. Whether it was exclusively through a vertical line of evolution how birds emerged from reptilian ancestry, and/or was it with the help of horizontally inserted genes, it will be determined by renewed further research. May be, the answers will come from the International Chicken Genome Sequencing Consortium (Washington University School of Medicine, Campus Box 8501, 4444 Forest Park Avenue, St. Louis, Missouri 63108, USA).

Is there a chain of herpesviral viral evolution in which ancestral reptilian and amphibian herpesviruses (the ancestor of the fibropapillomavirus of extant turtles) (*vide supra*) infected the dinosaurs? Before their extinction, the dinosaurs passed these viruses to the ancestors of *Aves*. Extant species of *Aves* are infected with the descendants of these ancestral herpesviruses (gallic herpesvirus 2, the MDV; psittacine cloacal papillomatosis herpesvirus). After the divergence of the reptilian/amphibian and the mammalian lineages, the mammalian species continued to harbor the descendants of the ancient reptilian-amphibian herpesviruses (the ancestor of the fibropapillomavirus of extant turtles) and expressed them up to the simian lineages. These herpesviruses are known as the baboon, green monkey, mandril and rhesus rhadinoviruses, the retroperitoneal fibromatosis herpesviruses. These ancient rhadino-herpesviruses (herpes, erpein, creepeing-creeper; rhadino, fragile viral DNA); already encode an IL-6 homolog and their LANA (latent nuclear antigen) is an ortholog of the HHV-8/Kaposi sarcoma HV ORF73 product protein (reviewed in [213]). The anti-apoptotic LANA proteins promote cells survival and "immortalization" [289]. The polymerase enzyme of the chimpanzee rhadinovirus shows 82% nucleotide sequence homology and 93% aa identity with the HHV-8/KSHV (but chimpanzees do not develop Kaposi sarcoma-like tumors) (reviewed in [213]). In KS cells HHV-8/KSHV activates the MAPK pathway (mitogen-activated kinase) [290]. The ORF (open reading frame) K12-product kaposin [291, 292], and other oncogenes of HHV-8 (v-Bcl-2; the ORF72 product v-cyclin D; the ORF74 product G-protein-coupled receptor, long unique region, cyclooxygenase-2) [293] are new acquisitions of KSHV (HHV-8), which are not yet present in the chimpanzee rhadinovirus (reviewed in [213]). In the latent form of HHV-8/KSHV, the viral genome is circularized in an epigenetic extrachromosomal location. Hypomethylation of the promoter of replication and transcription activator, and histone acetylation trigger the "lytic switch" and thus the active replication of the virus leading to cytolysis [294]. It is not only HIV-1-induced immunosuppression that activates the latent HHV-8/KSHV The HIV-1 *tat* gene product transactivator protein Tat activates the MAPK pathway in the host and the kaposin gene in the latent HHV-8/KSHV [295]. Kaposi sarcoma cells through human leukocyte antigen HLA-A2 express epitopes that attract cytotoxic lymphocytes. These are *aa* 16–25 in latent antigen kaposin and aa 59–68 in lytic antigen glycoprotein H. The lymphocyte donors were healthy volunteers [296, 297]. This author presented microphotographs of the phenomena of cytotoxicity by autologous lymphocytes to KS sarcoma cells in the pre-AIDS era [298–300]. The coexistence of the herpesviral pathogens (HHV-8/KSHV) and an as yet unidentified endogenous retrovirus in pre-AIDS era "classical" KS cells is shown in Fig. 2.3a,b. The pathogenicity-

and proto-oncogenes of HHV-8/KSHV very likely are host cell gene derivatives expropriated through horizontal transfers by the ancestral rhadinovirus as it was following the hominid lineage after the divergence from the *Pan* lineage some 7 million years ago. These horizontal new gene acquisitions by the ancestral rhadinovirus must have taken place during its residence in the ancestors of *Homo* (the *Australopithecus afarensis* and upward).

The ancestry of the other human "lymphocryptovirus," the Epstein-Barr virus (HHV4/EBV) can be traced back to New World marmoset (owl and squirrel) monkeys diverging from the Old World simian lineages some 33 million years ago and represented by the oncogenic *Herpesvirus saimiri* in its host the squirrel monkey, *Saimiri sciureus* [301]. In New World (South American) monkeys, in contrast to retroviruses dominating in Old World (African) monkeys, the lymphogenic viruses are herpesviruses [302]. Further down, in the Cambrian sea and at the time of the emergence of the ancestral sharks (Placoderms; *carcharhine* sharks, *chondrichthyes*, g*nathostomata*), an ancestral lymphocryptovirus might have been instrumental in inserting genes to encode the basic elements of adaptive immunity (*vide infra*).

2.4 Horizontal Gene Transfers in Archaea and Prokaryota

2.4.1 The Darwinian Threshold (Woese)

Many new viral genomes emerge from the sea (through metagenomics, *vide supra*), that encode proteins so far unknown in multicellular organisms ("viral hallmark genes") [303]. Thus, a "primordial virus world scenario" has been envisioned [304–306].

Some of the large dsDNA viruses of dinoflagellates replicate exclusively in the host cells' cytoplasm, where they create "virus factories" or "viroplasms" (*vide supra*). These events reflect back to the most ancient times at the origin of precellular and protocellular life, and to the forms in which predecessors of the protocells in the "virus world" existed ("Abiogenese der Virusarten. Eine weitere Möglichkeit der Entstehung der Virusarten ist, daß sie, aus leblosen Stoffen stammend, als erste Lebewesen auf der Erde erschienen") [180]. There, RNA viruses, then retroid elements (RNA → DNA), and then DNA viruses formed sequentially. A network of interacting nucleic acids segments existed before protocells were formed [303, 304]. Viruses pre-dating the origin of and not fitting into the tree of life of the cellular living formations [307], entered the first archaea, prokaryota and eukaryota cells from the outside. Or was it ancestral cellular genomes from which the first viral nucleic acid segments excised themselves ("Endogene Abstammung der Virusarten") [180]. Or was it intracellular bacterial symbionts, that through gene losses became what is now known as the *lymphogranuloma inguinale* virus, *molluscum contagiosum* virus, psittacosisvirus, trachomavirus ("die Abstammung der Viren von den Mikroben der Urzeit"), whose replication by fission of large "initial bodies" resembles that of pleuropneumonia bacteria, or L-forms ("in Elementarkörperchen zerfallende große Gebilde", "Plaquebildung", "Matrixmaterial"?) [180]. This is how the trachoma virus became *Chlamydia trachomatis.* As the large nucleocytoplasmic dsDNA viruses are replicating in "virus factories" or "viroplasms" exclusively in the cytoplasm (*vide supra*), they reactivate the idea that the first nuclei were formed in the large dsDNA virus-infected cells [308a,b, 309].

Protocells needed thymidylate synthetase enzymes (ThyA/ThyX) for the production of deoxythymidylate to build DNA. Rampant lateral transfers of these enzymes occurred between the three domains of ancient life and phages/viruses were enlisted as vectors [310]. It is well accepted and reviewed that fused archaea and prokaryota cells might have formed the first eukaryotic cells [311–313]. Ancient fusogenic viruses, like the ancestor of the extant mycoplasma phage MV-L3 (from *Acheloplasma laidlawiii*) might have mediated the first such unisons of *Crenarchaeota* and prokaryota spheroplasts [213, 314–316], since *Crenarchaeota* and eukaryota are evolutionarily related due to

similarities of their cell divisional machinery [317, 318]. This seminal experiment of Nature can be repeated in the laboratory today by fusing extant crenarchaeal and prokaryotic proto-spheroplasts with fusogenic mycoplasma viruses for the production of some primordial eukaryota-like cells [315, 316].

The rRNA studies of C. Woese aimed at the phylogenetic evolution of prokaryota lead to the discovery of archaea. Ribosomal proteins reflect backwards to the phylogenesis of the species. The universal ribosomal proteins functioned in coalesced protoplastic-spheroplastic cells prior to the separation of the phyla archaea and prokaryota. These cells exchanged large portions of their genomes by massive horizontal transfers. Speciation ("the origin of species") has taken its beginning when the first domain-specific ribosomal proteins appeared. At that point, the Darwinian threshold was established and the uncontrolled exchanges of genetic material became replaced by the rules and regulations of vertical inheritance. The 16S and 23S rRNAs established their sequence identity. Structural signatures of 16S and 23S rRNAs clearly distinguish archaea and prokaryota (bacteria), while the universal rRNA genes and proteins remain conserved and recognizable. The relative ordering of the universal r-protein genes within the rRNA gene cluster in archaea and prokaryota (bacteria) preserve their very extensive similarities, Hyperthermophilic archaea survive at 90°C. Archaea preserved their genomes by vertical inheritance, but were gene donors to prokaryota and eukaryota beneath the Darwinian threshold. The hyperthermophilic bacteria (*Aquifex aeolicus; T. maritima*) expropriated the archaeal genes of thermophily. The *Thermus thermophilus* megaplasmid is operational in *Deinococcus radiodurans*; if these two entities shared a common ancestor, it was through vertical inheritance, otherwise by horizontal gene transfer that they possess this megaplasmid. The archaeal stem diverged into the three lineages (*Crenarchaeota, Euryarchaeota, Nanoarchaea*) forming the first domain of life on the primordial Earth (prokaryota, second, and eukaryota, third). Euryarchaeota are methanogenic. The ribosomal superoperon consisting of some 50 cotranscribed and coregulated genes encoding ribosomal proteins is operational in both archaea and prokaryota. *N. equitans* lives as a parasite of the archaeon *Ignicoccus hospitalis* [319–323]. In the genus of Neisseria, widely dispersed genomic clusters identical with those of other Neisseria species and other bacterial genera, suggested to Maynard Smith that the excess of horizontal gene transfers obliterated speciation and that "there are no such entities as species in these pathogenic bacteria" [324, 325].

Horizontal gene exchanges between ancestral archaea and prokaryota were so pervasive that the early evolution of cells did not follow the vertical outbranchings of a Darwinian phylogenetic tree. Thereafter the vertical outbranchings of the Tree of Life gained dominance and continuing horizontal gene transfers failed to significantly alter the course of evolution. The central trend prevailed undisturbed by random horizontal gene transfers. At and after the level of the radiation of archaeal and prokaryotic phyla, the central trend representing vertical inheritance remained quite discernible [326].

There are examples of horizontally (laterally) occurred gene acquisitions for practically all bacterial functions (photosynthesis, aerobic respiration, nitrogen fixation, sulfate reduction, methylotrophy, isoprenoid biosynthesis, quorum sensing, flotation on gas bubbles, thermophily, and halophily) [327]. The EMBO Conference on Molecular Microbiology, Heidelberg, 2006, discussed noncoding regulatory RNA, RNases and gene expression, genomics, evolution and bacteriophages, signal transductions, protein interactions and networks, pathogenicity, virulence and endosymbiosis, chromosome dynamics, DNA uptake, and other subject matters. These presentations were published with extensive literature quotations [328]. Here is the background and the environment in which horizontally transferred genes are released and accepted. The PLoS Genetics review specializes on those horizontal gene transfers in prokaryotes that accelerated the evolution of the recipients [329] (*vide supra*). One example is the lack of lysine biogenesis pathway in "the last common ancestor of life" and the acquisition of this pathway by ancestors of *Crenarchaeota, Deinococcus-Thermus* and *Pyrococcus* occurred through horizontal gene transfer from prokaryota. The prokaryotic genomes evolved rapidly due to horizontally transferred genes of whatever means of transfer, other than vertical [323].

Reverse gyrase of the hyperthermophilic archaeon, *Sulfolobus acidocaldarius* (*vide supra*) is shared between the archaeal and the bacterial (prokaryota) ancestors of hyperthermophiles,

Thermotogales and *Aquificales*. These genes have been widely dispersed between archaea and bacteria through routes of horizontal transfers [322]. The *Thermotogales* genomes reflect well to events predating the Darwinian threshold. The composition of the genomes of *Thermotoga maritima* (Ttm) and *Aquifex aeolicus* (Aa) (*vide supra*) places them together at the base of the bacterial tree, in the era when the construction of a phylogenetic tree based on strictly vertical transmission of genes was invalidated by pervasive horizontal gene transfers. So far no prophages were found in the Ttm and Aa genomes. Some remnants of phage gene sequences in the thermotogales genome were discovered when sequences related to an *E. coli* phage showed up within two thermotogales genomes. *Thermotogales* bacteria were already armed with the antiviral related CRISPR elements (*vide supra*). Hyperthermophilic archaea (*Thermococcales* and *Pyrococcus furiosus*) horizontally exchanged their CRISPR elements [330]. In the absence of phages, horizontal gene transfers in thermotogales were not likely to be virally mediated. The genes securing life at high temperatures are shared with hyperthermophilic archaea. All thermotogales share closely related rRNA genes; rRNA analysis proves monophyly for Ttm and Aa. The operon for the membrane-associated proton-pump ferredoxin oxidoreductase is an archaeal feature; a derivative of it shows up in *Pyrococcus furiosus*. A derivative of the thermotogales ruBisCO gene, or rubisco-like proteins (*vide supra*) are operational in *Bacillus subtilis*. Derivatives of the thermotogales genes for the methionine salvage pathway are present in some deep sea bacteria. The first protocells might have been thermophilic and later life forms lost thermophily, even in some thermotogales with a change from ancestral to later 16S rRNA variations [331]. Thermotogales genomes show incongruent evolutionary history dating back to the pre-Darwinian threshold era.

The aminoacyl-tRNA synthetases secure the fidelity of protein synthesis as specified by the mRNAs. The aminoacetylated amino acids are attached to the 3′-ends of cognate tRNAs. The anticodons of the aminoacyl-tRNAs specifically react with the trinucleotide codons of the mRNA. The recognition of amino acids (aa) is quite specific. The anticodon GUC for aspartate is different from anticodon GUU for asparagine in the crenarchaeon *Sulfolobus* [332]. These ancient enzymes converted the RNA world into the protein world. These sequences of the aminoacyl-tRNA synthetases (aaRS) evolved by gene duplications, horizontal gene transfers and genetic recombinations. It appears that some aaRS genes readily crossed between Archaea, Eubacteria (Bacteria) and Eucarya (Eukaryota). Other aaRSs are individually analyzed and their phylogeny reconstructed. Duplications, fusions, recombinations and horizontally executed exchanges of the aaRS genes exhibit great diversity. While the majority of aaRS genes can be fitted into the phylogenetic pattern of vertical transmission within the three domains of life, some individual aaRS genes defy the rules of vertical transfers. For example, the yeast mitochondrial PheRS is related to that of *H. influenzae* and *Synecoccus* [333]. Crystallographic studies of the *Pyrococcus horikoshi* tyrosyl-tRNA (TyrRS) and tryptophanyl-tRNA synthetase (TrpRD) indicate that the original TyrRS was the ancestor of TrpRS in archaea, and that from archaea the TrpRS was horizontally transferred to bacteria [334].

A phototrophic filamentous microbial community existed in the Buck Reef Chert along what is now the South African coast 3.4 billion years ago. This population fixed CO_2 in the Calvin cycle and received electrons from atmospheric hydrogen [335]. These microorganisms were the ancestors of cyanobacteria already practicing anoxygenic photosynthesis. Their reaction center 1 (RS1) reduced nicotinamid adenine dinucleotide phosphorylated $(NAD(P)^+$ to NAD(P)H. In starvation these cells fixed nitrogen. Upon acquisition of photosystem II (PSII) and RS2 (able to oxidize water) over the pre-existing PSI and RS1, these procyanobacteria transgressed the Darwinian threshold and evolved into oxygenic photosynthesis conducting extant cyanobacteria. Cyanobacterial photosynthetic genes (gene clusters and gene product proteins, the core cyanobacterial clusters of orthologous groups of proteins, core CyOGs) spread either by horizontal transfers in the contemporaneous living world: into the green sulfur bacterium *Chlorobium tepidum*, the green nonsulfur bacterium *Chloroflexus aurantiacus*, the Gram-positive phototrophic bacterium firmicute *Heliobacillus mobilis*, and the purple α-proteobacteriun *Rhodopseudomonas palustris*. Endosymbiotic cyanobacteria in plastid-carrying

eukaryotes, the apicomplexans, the vestigial plastid-carriers (*Plasmodium falciparum*), diatoms (*Thalassiosira psudonana*), and algae (*Cyanidioschyzon merolae*), and from algae transferring eventually into plants occupying dry land (*Arabidopsis thaliana, Oryza sativa*, etc), propagated further the chlorophyll- and phycobilin-based photosynthetic genome. Protective chlorophyll-binding proteins dissipate excess light energy and thus secured the survival of cyanobacteria [336, 337].

Beneath the Darwinian threshold, and later transgressing it, pro-cyanobacteria and their phages coexisted. Phages form the bacterial "mobilomes." The oceanic prototroph, *Prochlorococcus,* carries myo-, podo- and siphoviruses (named after their morphology; for example the member of tailed phages, *Caudovirales, Siphoviridae* possess long noncontractile tails). The myo- and podoviruses propagated their structural genes to T4/T7 coliphages. In addition, these *Prochlorococcus* phages carry cyanobacterial photosynthesis genes. In contrast, the siphovirus lacks the cyanobacterial photosynthetic genes, but exhibits 14 other cyanobacterial homologue genes. Lytic cyanobacterial phages replicate in the bacterium and egress by bursting it. Temperate phages insert their DNA genome into the host bacterial chromosome and as prophages replicate with the host genome. Temperate phages carry out horizontal gene transfers, including those genes that encode pathogenicity islands and toxin genes (*vide infra*). However, most cyanobacterial isolates are devoid of prophages (and pathogenicity islands and toxins) [338, 339]. Cyanobacteria of the genus *Synecoccus* are infected by the phage, cyanomyovirus S-PM32. This phage shares some of its structural proteins with coliphage T4, its other structural proteins are unique and unrelated to other phages [340]. Phage homing endonucleases encoded by the endonuclease genes perform site-specific DNA cleavage. The cyanobacteriophage S-PM2 endonuclease is homologous to the resolvase of coliphage T4. Group I introns (inteins) can disrupt the DNA recognition site of the endonuclease. Otherwise the endonuclease makes a specific double strand cut of the DNA. The endonuclease can not cleave the introns-containing core photosynthesis reaction center gene (*psb*A). Mobile introns in the same target sequence that the endonuclease attacks, protects the host cell genome. Collaborative homing introns protected the photosynthetic genes [341].

Ancient bacteriophages are the ancestors of all viruses [342]. Some archaeal prophages (*Sulfolobus* turreted icosahedral virus in a crenarchaeal host; and others integrated into the tRNA of euryarchaeal hosts) are the ancestors of eukaryotic adenoviruses [343]. While temperate phages are accepted, even welcome for the useful genes they might insert, lytic phages are opposed for the life of the host. In this most ancestral virus-host relationship, the host genomes arranged the clustered regularly interspersed short palindromic repeats (CRISPR), the small RNA-guided defense system in archaea and prokaryota. The system prevails in eukaryotic genomes as well. Halophilic archaea defended themselves against lytic phages by a highly conserved CRISPR mechanism [344]. The first use of this defensive system must have occurred beneath the Darwinian threshold. In prokaryota the use of the system transgressed the threshold [345–348]. Bypassing vertical inheritance, the valuable gene clusters of the CRISPR system were acquired by horizontal transfers mediated by phages and plasmids [349]. Beyond the Darwinian threshold, prokaryotic genera widely accepted the horizontal transfer of, and applied the CRISPR defensive system against phages and plasmids, including the universal *cas* 1 gene. Same in eukaryotic cells, the system targets the nucleic acid of the invaders in a sequence-specific manner. In response, point-mutated viral genomic sequences escape recognition by the CRISPR system; a massive viral invasion of a cell could overwhelm the CRISPR system [350].

2.4.2 The Ancient Origin of "Virulence Genes"

Salmonella typhimurium and *E. coli* acquired multiple virulence genes through horizontal transfer mediated by phages and plasmids [351]. These gene product proteins enabled ancient bacteria to thrive under "adverse circumstances," The sites of residence for these genes are the "pathogenicity islands."

When the bacteria infect a host and thus encounters "adverse circumstances," it is the activation or acquisition of "pathogenicity islands" that secures the new life style of the invader. These complex gene cluster transfers appear to have developed after speciation occurred above the "Darwinian threshold." These gene clusters are transferred by conjugation or by phage transduction. However, intruding phages and plasmids encounter the CRISPR/CAS defensive system (*vide supra*). The CRISPR/CAS system can eliminate horizontal gene transfers conducted either by conjugation or phage transduction. While it protects bacteria from lytic phages, it may deprive them from the acceptance of genes beneficial to them in "adverse circumstances." A CRISPR system prohibits the acceptance of staphylococcal conjugative plasmids in clinical isolates of *Staphylococcus epidermidis* [352].

Most of the virulence genes of *E. coli* were acquired during the last 100 million years through horizontal transfers [353]. The uropathogenic *E. coli* acquires its pathogenicity island genes (fimbria, adhesins, LPS, toxins, hemolysins, siderophores) through horizontal transfer and in its biofilms and within bacteria-loaded epithelial cells it commonly overcomes both innate and adaptive immune reactions of the host [354, 355]. In the Netherlands, Enterobacter strains (*E. cloacae, E. hormaechei)* may be one of the sources of the pathogenicity island genes of uropathogenic *E. coli* strains [51] (*vide supra*). The high pathogenicity island (HPI) gene cluster may be transferred also to *Klebsiella pneumoniae* strains. Yersiniabactin (from *Y. pseudotuberculosis*) is such a HPI. The HPIs contain integrases, an iron uptake system (a catecholate siderophore) and an integrative and a conjugative element (ICE) with genes producing enzymes for excision and integration for DNA conjugative transfer. Some ICEs do, others do not contain helicase encoding DNA [51]. In Hungary, *Klebsiella pneumoniae* strains acquired plasmids up to 230 kb in size. The ciprofloxacin-resistant VTX-M-15-producing *K. pneumoniae* strains (VTX, from verocytotoxigenic *E. coli*) spread in an epidemic fashion in six nosocomial outbreaks [356]. In Galveston, TX, fluoroquinolin-resistant uropathogen *E. coli* emerged to infect renal transplant patients; these "virulent appearing" *E. coli* strains remained susceptible to 3rd generation cephalosporins [357]. The EMBO conference on prokaryotic genomic evolution and gene expressions [328], dealt with the noncoding regulatory RNAs of *E. coli*, one of which, a sRNA, suppresses the synthesis of toxic peptides [358]. Could this mechanism neutralize toxin production in a HPI?

Acquisition of HPIs is usually combined with antibiotic resistance. The genes for antibiotics production in streptomyces fungi, for antibiotics resistance and for pathogenicity island acquisition in bacteria are received through horizontal transfer by plasmids or by bacteriophages. *Enterococcus faecium* existed as an avirulent commersal until after it developed a new surface antigen Esp, and acquired a collagen adhesion gene and gene product protin, It has become a multidrug-resistant pathogen [359, 360]. Some strains of *E. faecium* received a large pathogenicity island containing the virulence genes *esp* (enterococcal surface protein), *hyl* (hyaluronidase), *acm* (collagen adherence) and genes for cytolysin and exotoxin. *E. faecium* and *E. faecalis* acquire these genes from "another common source." The CRISPR-CAS system (clustered regularly interspaced short palindromic repeats, C; C-associated) is non-functional in these enterococci. *Siphoviridae* bacteriophages enter the bacterial genome uninhibited [361]. Thus, these genes circulate now in colonies of streptomyces fungi and bacteria. Just when the original genes were generated in the ancient communities shared by the ancestors of prokaryotes and streptomyces, or after these taxa and genera diversified, remains to be resolved.

The mobile genetic elements (DNA transposons), plasmids and phages serve as vectors of the virulence genes of pathogenicity islands; repeated insertions may unite individually transferred genes into the whole of a pathogenicity island. The virulence gene-product proteins (*rpsl* genes, ribosomal protein subunits L) reside in the donors' ribosomes. Among others, *Streptococcus agalactiae* and *Serratia marcescens* possess such *rpsl* (*rpl*S) genes. At the dawn of combination chemotherapy, patients with acute leukemia succumbed to *Pseudomonas aeruginosa* septicemia in 8–24 h. The pseudomonas pathogenicity island (PAP) of 115 genes is first excised from the donor's chromosome. Then a 10 gene cluster (related to an enterobacterial plasmid) with the help of a prepillin peptidase conjugation system, transfers through a type IV pilus to another *P. aeruginosa* strain [362]. The antibiotics

resistance-encoding genes are transferred either by conjugation (STX, *vide supra*), or by means of horizontal transfer by plasmids or phages.

Conjugative plasmids transfer bacteriocins. Circular bacteriocins are linked at their N- and C-terminal ends. Could the *Enterococcus faecalis* circular bacteriocin, enterocin [363–365] be used to kill HPI-expressing enterococci (*vide supra*)? All these transfers, especially those of bacteriocins, show species-specificity. Thus, the genes encoding these systems (antibiotics production; antibiotics resistance; HPI acquisition; bacteriocin production) must have evolved above the Darwinian threshold, after speciation, when rigidly controlled vertical inheritance established itself[2].

2.4.3 A Selected Example of Speciation

Mycobacterium marinum is a close relative of *M. tuberculosis* in that they derive from a common ancestor. *M. marinum* possesses a 6,636,827-bp circular chromosome with 5,426 coding sequences of which 23 are nonribosomal peptide synthases and 18 are of unknown function and without orthologous genes in other mycobacteria. The early secreted exported antigenic targets (ESX1-6; SX-A/B; ESAT) ATP-dependent system is encoded from 29 *esx* genes; this gene number is reduced to 23 in *M. tuberculosis*. The ESX expressions relate to virulence, intercellular spread, induction of granuloma formation and to the ability to grow in vitro. The ESX proteins activate inflammasomes through induction of IL-1β and IL-18 secretion. The ESX-5 protein suppresses the production of IL-12, TNF-α, and IL-6. The *mel*2 locus (*mel* loci confer enhanced infection; *mrl* loci confer repressed infection) encodes proteins protecting *M. marinum* in infected macrophages from reactive oxygen and reactive nitrogen species (ROS; RNS). Bacterial cell wall lipooligosaccharides suppress TNFα production in infected macrophages. These data derive from *M. marinum*-infected mouse and human macrophages, not from infected fish [367–370]. *M. marinum* harbors the genomes of 10 prophages, and a 23-kb mercury-resistance plasmid. Some of the large repertoire of non-ribosomal peptid synthase genes of *M. marinum* were acquired horizontally. *M. marinum* and *M. tuberculosis* share 3,000 orthologs with amino acids identity of 85%. The large genome of *M. marinum* provides for its extensive host range and its ability to survive in its aquatic environment. The downsized genome of *M. tuberculosis* restricts its host range; it is adequate for its intracellular life style; the acceptance of horizontally acquired genes provides for its survival in an immunologically active host [371]. There are 695 coding sequences (CDS) present in *M. marium* and missing from *M. tuberculosis.* Of the remaining CDS,

[2]This author at the end of December, 1956, upon his introduction as a Rockefeller fellow to Professor Selman Waksman, the Nobel-laureate discoverer of streptomycin, and director of the Waksman Institute, at Rutgers, the State University of New Jersey, New Brunswick, N. J., was immediately asked to elaborate on the mechanisms how streptomyces fungi in the soil acquire the potency of antibiotic production. He answered that antibiotic production must have occurred as a response to challenge, as bacterial and fungal species competed for space (“niche”) and nutrients. Dr. Waksman said: “but you failed to show this in an experiment you have published.” This astonished fellow was speechless: “will the professor cancel my fellowship”? Dr. Waksman was referring to a paper published the year before in German (J. Sinkovics: “Untersuchungen über die Wechselwirkung nicht-antibiotischer Pilze und Bakterien.” [366]), in which no antibiotics appeared in the fluids of common cultures of fungi and bacteria. It was amazing that he knew about it. Most generously Professor Waksman did not cancel the fellowship for this fellow’s failure to generate antibiotics production at will. If asked the same question today by his students, this author would answer: “In my cultures there were no antibiotics-encoding gene donors for the fungi; these ancient genes are not generated anew, in extant fungi and bacteria they are acquired from donors by horizontal gene transfers mediated by mobile DNA elements (transposons), plasmids and phages. The same principles apply to antibiotic resistance genes and pathogenicity island genes. These latter genes serve bacteria to survive under ‘adverse circumstances.’ Amoeba, unicellular protists and some nematodes (Caenorhabditis) feed on bacteria and win. It is a great misfortune to advanced multicellular organisms that they present themselves to bacteria as ‘adverse circumstances.’ The bacteria must acquire and activate their pathogenicity island and antibiotic resistance genes to survive and thrive in these hosts. No offense meant.” The students would be disappointed with this answer and would look up better answers in Internet/PubMed.

80% are orthologs between *M. marinum* and *M. tuberculosis*. Native *M. marinum*, the causative agent of fish tank granuloma in the human host, can be eliminated by healing and can be effectively treated with antibiotics (rifampin, ethambutol, clarythromycin and others). This author wonders if patients with healed fish tank granulomas acquire some immunity against *M. tuberculosis*?

The virulence operon Rv0986-8 of *M. tuberculosis* was transferred by a plasmid of gamma-proteobacterium derivation [372–374]. Laterally (horizontally) acquired genes in *M. tuberculosis* are in loci encoding sulfolipid metabolism, lipid glycosylation, adhesins, pilin development, fumare reductase synthesis in anaerobiosis, molibdopterin synthesis for nitrate respiration in reduced oxygen tension, such as within granulomas. *M. tuberculosis* engages 250 of its genes in fatty acid metabolism. Some of these genes (gene families) originated from actinobacteria [375]. The direct repeat region (DR) of *M. tuberculosis* contains 30-bp repetitive sequences and spacers with genes encoding proteins of unknown function. These sequences are absent in micobacteria (*M. smegmatis*; *M. avium*) that are philogenetically older than *M. tuberculosis*, therefore these segments were not vertically inherited, but horizontally acquired from an unknown source [376]. Thus, the DR including the CRISPR locus for providing resistance to bacteriophages, is another horizontally acquired operon. The original CRISPR in prokaryota were synthesized by the bacteria as modified phage genomic sequences placed as "spacers" in the bacterial genome [377].

The PE/PPE (PE = Pro-Glu; PPE = Pro-Pro-Glu) acidic glycine-rich subgroups of recombinant proteins (PGRS) are encoded by a family of genes to occupy positions on the bacterial surface. There, they induce host B cell- (the ORF Rv2430c derivative) and T cell- (the Rv2608 gene product) mediated immune reactions [378–380]. The PPE gene regions are hypervariable [381, 382], that is able to alter surface antigens under the pressure of the immunoreactive host.

The *M. bovis*-derived Bacille Calmette Guérin (Pasteur Institute BCG; BCG Denmark; BCG Tice & Glaxo USA) has been distributed around the world as far as to Russia and to the Orient (BCG Russia; BCG Beijing; BCG Tokyo). BCG Tokyo retained its full size of 4,371,711 bp containing 4,033 genes, of which 3,950 encode proteins. Retention *versus* losses of the original genes varies in the foreign passage lines of BCG strains. For example, BCG Tokyo/Japan retained its trehalose 6,6′dimycolate (TDM) production, and thus induces strong IL-12, IFN-γ, TNF-α response, whereas the BCG Connaught lost parts of its TDM production and therefore is a weak inducer of Th1-type immune reactivity. In India, weak cytokine-inducer BCG treatment of bladder cancer resulted in higher rates of relapses. Some BCG strains induce more the tolerizing cytokine IL-10, than the Th1-type cytokine IFN-γ [383–387]. A mutated narK2X promoter in its 110 region deprives *M. bovis* of the nitrate reductase enzyme. This enzyme in *M. tuberculosis* is vital for bacterial survival under hypoxic/anaerobic conditions, which prevail within granulomata [388], thus reducing BCG's pathogenicity. Horizontal gene implants (perfringolysin; MUC1 mucin and granulocyte-macrophage colony stimulating factor, GM-CSF) into the genome of BCG strains intensifies the vaccines immunogenicity [389, 390]. The multiple drug-resistant Beijing *M.* tuberculosis strain emerged as "an evolutionary response to BCG vaccination against, and antibiotic therapy for" tuberculosis [391]. Ancient records of paleopathology reveal that human tuberculosis predated that of domesticated animals (bovine tuberculosis) [392] Tuberculosis decimated mankind 35,000 years ago (or much longer).

The strictly intracellular parasite, *M. leprae* enters Schwann cells through α-dystroglycan-laminin cell surface complex. The same entry site is used by some arena viruses (Lassa virus, lymphocytic choriomeningitis virus). The Schwann cell invaded by *M. leprae* either dies in apoptosis, or proliferates. Cell proliferation is induced by the activated p56Lck (lymphoid cell kinase), a Rous sarcoma virus-related kinase (*src*) gene-product proteins. In surviving Schwann cells, NFκB translocates into the nucleus. These cells release solubilized receptors of TNF-α, but infected myelinating Schwann cell can not produce myelin. If myelin protein P0 (Pzero) is produced, *M. leprae* binds to it [393]. Activated macrophages surround the lesions and phagocytose, but fail to kill the bacteria. In Lucio's phenomenon, extensive vascular endothelial cell necrosis occurs, numerous skin ulcers develop and contain large foamy macrophages loaded with bacteria (*M. leprae*). Leprosy becomes a

chronic ailment in patients with Th2-type immune reactions (producing IL-10 and TGF-β). Patients with lepromatous leprosy generate regulatory T cells (T_{regs}) suppressing Th1-type immune reactions (reviewed in [213]). The intracellular NOD system (nucleotide-binding oligomerization domain) activates the first innate immune reaction in the host; single nucleotide polymorphisms render the NOD system deficient, increase the susceptibility upon exposure and direct the disease to advance into the multibacillary stage instead of the paucibacillary stage [394]. Lectins of *M. leprae* and *M. tuberculosis* activate in DCs the signaling pathway of proto-oncogene *raf*-1 (rat fibrosarcoma) and the Raf-1 protein acetylates NFκB subunit p65 after NFκB was already induced IL-10-mediated Th-2 type environment [395]. Thus, *M. leprae* activates proto-oncogenes (*src-p56lck, raf*-1) to induce host cell proliferation, or host immunosuppression, but without malignant transformation of the invaded cells.

Mycobacteria (*M. tuberculosis,* BCG, *M. leprae*) induce immune T cell- ($CD4^+/CD8^+$-) mediated reactions in the human host. The immune T cells are polyfunctional secreting more than one lymphokines/cytokines (IFN-γ, TNF-α, CD107a, macrophage inflammatory protein-1β). Patients with sarcoidosis produce similar immune lymphocytes responding to *M. tuberculosis* antigens ESAT-6 and katG (Elispot-associated antigens; catalase-peroxidase) [396, 397].

In contrast to the variability of BCG strains maintained in laboratories worldwide, the naturally dispersed strains of *M. leprae* originally penetrating Europe through the Silk Road from the Orient and remained stable. Brazilian, Indian, USA (Mexican) and Thailand strains of *M. leprae* share 99.995% sequence identity [398]. Single nucleotide polymorphisms were interpreted as resistance to gene mutations and to horizontal gene insertions. Variable number of tandem repeats (VNTR) distinguishes four genotypes of *M leprae* based on single nucleotide polymorphism (SNP) [399]. While *M. leprae* lacks the ability to grow in laboratory media, it can infect the nine-banded armadillos in the Southeastern USA from Texas to Florida [400]. In the laboratory *M. leprae* can be grown in the foot pads of nude rats. *M. leprae* DNA/RNA extracted from such tissues so far did not reveal the acquisition of host cell genes; instead, *M. leprae* eliminated many of its resident genes by silencing them, and converting them to nonprotein encoding pseudogenes. Patients with leprosy yield large numbers of bacteria from their nasal smears. The *M leprosy* genome contains 1,514 ORFs and 1,133 pseudogenes; the *M. tuberculosis* genome contains 278 pseudogenes [401, 402].

A new isolate from a patient with lepromatous leprosy significantly differs from *M. leprae;* it is a new species by detailed genetic analysis. *M. lepromatosis is* closely related to *M. leprae.* The divergence of *M. leprae* and *M. lepromatosis* is estimated to have occurred 10 million years ago. *M. lepromatosis* was being isolated and studied at M. D. Anderson Hospital, Houston, TX [403]. In this hospital, patients with leprosy usually coming from Mexico [404, 405] are periodically encountered and attended, either granulomatous diseases were mistakenly considered to be malignant tumors, or Lucio's phenomena erupted when a patient with a *bona fide* malignant tumor (a sarcoma) and latent leprosy received chemotherapy [406].

In the course of the mycobacterial speciation, the strictly intracellular parasites (*M. leprae; M. tuberculosis*) constrict their genomes rather than expanding them by horizontally acquired new genes. *M. ulcerans*, the causative agent of the devastating Buruli ulcer in Africa, is responding to rifampin and streptomycin therapy [407]. *M. ulcerans* diverged recently (in evolutionary terms) from the *M. marinum* lineage to become a "niche-adapted specialist;" as such, it is undergoing continuous genomic reduction [408]. By variable number tandem repeats (VNTR) typing. *M. ulcerans* strains diverged further into substrains [409]. *M. ulcerans* now exists in two distinct lineages. The ancestral lineage emerged and spread from South East Asia (China and Japan) to South America including Mexico. The classical lineage emerged and spread in South East Asia, Australia and Africa. Genome reduction is more advanced in the classical lineage [410]. In Australia, Aedes mosquitoes and other carnivorous insects (*Naucouris* sp.) harbor *M. ulcerans* bacteria [411–413].

A mycobacterium isolated from frogs (MU128F) produces mycolactone toxin slightly different from the toxin produced by human-pathogen *M. ulcerans* (MUAgy99) [414]. The *M. ulcerans* 5,632 kb chromosome contains 771 pseudogenes. The 174 kb virulence plasmid produces the

polyketide toxin mycolactone [415]. Mycobacteria producing the ulcerogenic and immunosuppressive mycolactone (MPMs) originally deriving from laterally moving virulence plasmids (from *M. marinum* to *M. ulcerans*), by now due to shared common ancestry, preserve the gene by vertical inheritance [416]. The plasmids, the 174 kb pMUM001 and the megaplasmid of the 190 kb pMUM002 of *M. ulcerans* contain three *mls* genes. Non-mycolactone-producer *M. marinum* strains accept artificially created shuttle vector-mediated transfer of these genes, which encode polyketide synthetases and the toxin [417, 418]. *M. marinum*-derivatives *M. ulcerans* strains producing mycolactones (MPMs) infect fish and frogs in marshlands of the USA, and in the Red and Mediterranean seas. It is highly possible that the ancestral *M. marinum* acquired the original mycolactone genes from one of its ancient hosts (an amphibian, a frog). The genes encoding the toxins reside in horizontally spreading plasmids [419].

Natural horizontal gene gains occurred in *M. avium paratuberculosis* from soil dwelling proteobacteria and actinobacteria. Three mycobacterial genes, one of them the sigma factor regulator, show sequence similarity with some eukaryota genes [420]. *M. abscessus* gained horizontally transferred genes (phospholipase C; ABC Fe^{3+} transporter) from actinobacteria (*Rhodococcus*) and streptomyces sp. Non-mycobacterial genes of *M. abscessus* were acquired from *Pseudomonas aeruginosa* and *Burkholderia cepacia* in co-infected patients with cystic fibrosis [421].

2.5 The Insertion of Adaptive Immunity Genes

2.5.1 Retrotransposons

The innate immune system is based on antiviral (iRNA, siRNA, CRISPR, *vide supra*) and antibacterial defensive mechanisms, to which protection against invasion by foreign cells was added (like the first NK cells in the *Botryllus*) [422, 423]. The system consists of Toll-like cell surface, and NOD-like (nucleotide-binding oligomerization domain) intracellular receptors; phagocytes, monocytes and macrophages, the ancestors of dendritic cells, residing in coeloma cavities, or circulating in the hemolymph. Chemokines and cytokines work with the Toll-like signaling receptors, while it is not clear just exactly when the first alpha-beta interferons (IFN-αβ) were produced. Humoral immunity was, and still is, practiced in the lamprey and hagfish by the generation of antigen-reactive leucine-rich repeats in the variable lymphocyte receptors. The variable lymphocyte receptors (VLR) are hypervariable and occupy the concave surface of the structure. The secreted hypervariable receptors appear in dimers, tetramers and pentamers to bind highly specifically the targeted antigens in the extracellular spaces [424–427]. In cyclostomata fish (hagfish and lamprey), the VLR gene-carrier lymphoid cells undergo clonal expansion in response to antigenic stimulation, and encode a great diversity of leucine-rich repeats (LRR), which react with different antigens. The LRRs are not immunoglobulins.

The second system of adaptive immunity emerged in ancestral sharks hundreds of million years ago. One of the basic doctrines of the adaptive immune system is the preservation of the faculties of the entire innate immune system and cooperation with them in a mutually dependent fashion. This principle manifests itself best in the fundamental functions of the innate dendritic cells and natural killer (NK) cells in the adaptive immune system. Inhibitory NK cell receptors recognize the self major histocompatibility complex (MHC) molecules and the NK cell remains silent. Cells with down-regulated MHC molecules (virally infected cells; malignantly transformed cells) are recognized by the killer receptors (KIR) of NK cells and are attacked. In mammals, inhibitory and killer NK cell receptors are encoded from different chromosomes. Human KIRs are encoded from the leukocyte receptor complex (LRC) of genes [428]. In the adaptive immune system, histocompatibility antigens are recognized; antigen-presenting cells educate T lymphocytes for the induction of Th1-, Th2-, Th17-type inner immune environment in the host. The most intricate specific antibody production

(immunoglobulins, Igs) by B (bursal) lymphocytes maturing into plasma cells is the exclusive property of the adaptive immune system. T lymphocytes release most of the interleukins and cytokines; B lymphocytes through processes of somatic hypermutations construct the antigen-specific light chains of the immunoglobulin molecules. See original research [429–431] and reviews [431, 213].

Ancestral elements of the adaptive immune system existed singly, the V (variable), J (joining) and C (constant) regions in the protochordates, and an ancestral complement and both RAG1,2 elements (recombination activating genes) in the amphioxus (*Branchiostoma floridae*) but certainly in the sea urchin (*Strongylocentrotus purpuratus*) [432–434]. The VJ elements are the distant precursors of the B- and T-Lymphocyte receptors. These dispersed elements found each other in the placoderm sharks and in their descendants and started to work for the first time in a strongly regulated unison (reviewed in [213]).

The genetic recombinations of the V(D)J (variable, diversity, joining) elements and the single constant (C) region lead to specific antibody production. The system is activated by the *rag*1 and *rag*2 → RAG1,2 gene product proteins and regulated by the 9 bp (nonamer), 7 bp (heptamer) RSS recombination signal sequences. Recombinations of 12 and 23 bp take place between the heptamer and nonamer subsequences of RSSs; spacers separate the heptamer and nonamer RSS subsequences. The V and J segments recombine into a light chain coding region. The rearranged VJ segment is transcribed into a mRNA. Splicing of RNA removes the introns and the native J region. The light chain protein is arrived at by translation of the mRNA [435, 436].

Before the appearance of jawed sharks (chondrichthyes, gnathostomata), no creatures of the sea possessed this system working in unison. The sea urchin (*Strongylocentrotus*), sea anemone (*Nematostella*), lancelet (*Branchiostoma*), the mollusks, sea slug (*Aplysia*) and the hydra (*Hydra*) all possess and operate *rag*1 genes, but not for V(D)J somatic hypermutation activation. However, in the sea urchin, *rag*1 and *rag*2 already co-exist with the zink finger domain in *rag*1 fully operational. Some innate immunological function is assigned to RAG1,2 in the sea urchin, inasmuch as these gene product proteins are expressed in coelemocytes, the sea urchin's defensive cells. Coelomocytes respond to LPSs. The responding coelomocyte receptors 185/333 are capable of nucleotide sequence variations as the 185/333 genes encode similar proteins, but with a high level of sequence diversity [437]. These are the first immunological diversity (D) responses so far recorded (but these proteins are not immunoglobulins). Sea urchin coelomocytes also express the vertebrate complement components B and C3; these opsonize targets for phagocytic engulfment. In contrast, all other elements of the adaptive immune system are absent in sea urchins: there are no lymphocytes, no immunoglobulins and no B or T cell receptors. Crystallographic studies show structure similarities between sea urchin and vertebrate RAG1, 2. These findings may be interpreted so, that the *rag*1, 2 sequences were not newly inserted into the ancestors of he sharks, they might have been acquired at the sea urchin level. However, evolutionary inheritance lines in one direction toward echinodermata and the other line toward cartilaginous fish (chondrichthyes) diverged 400 million years ago.

Insect (drosophila, anopheles) transposases (transib transposases) are also similar in structure to the Rag1 proteins [438]. Mobile DNA elements have inverted terminal repeats (TIRs) similar to RSS and encode DNA-reactive enzymes [439]. The transposon N-RAG-TP of the sea slug (mollusk) *Aplysia californica* encodes a protein with its N terminal part being similar to that of the vertebral RAG protein. Transposon N-RAG is distinct from the transposons transib of other invertebrate species. The other transposon, similar to *rag1* is transposon Chapaev [437]. Some bacterial integrases are related to *rag* gene product proteins. The inverted repeat structure and the left/right asymmetry of the RSS elements are like the end structures of terminal repeats of the insertion sequences in bacterial genomes, when mobile DNAs encode transposases. A *rag* gene passage from *Bacteroides* to *Porphyromonas* has just recently been recognized [440]. The recombination of RSS-flanked DNA sequences is brought about by the *rag* gene pair encoding recombinases. These recombinases excise the RSS-flanked DNA and catalyze its transposition in a "cut and paste" manner. The *rag*1 and *rag*2 genes have a tight genomic linkage. Probably they had to travel together. The structure of the *rag*2 gene differs from that

of the *rag*1 gene. The *rag*2 is a eukaryotic gene. RAG2 may be an activator of RAG1. Indeed, RAG2 switches the catalytic center of RAG1 into its active conformation [441, 442].

The somatic hypermutations create millions of new genes encoding the antigen-specific immunoglobulin light chain molecules, or the configurations of the T cell receptors. Since there is no straightforward vertical inheritance lineage recognized between the lower sea animal-carriers of the RAG/RSS elements and the sharks, unless their common deuterostome ancestor already possessed the *rag*1,2 genes, these events of specific immunoglobulin synthesis and T cell receptor conformation in the sharks and above suggest that retrotransposons inserted through a horizontal route the V(D)J/RAG/RSS elements in unison or sequentially into the genome of ancient sharks. The aplysia (*vide supra*) *rag*1 gene resembles the N-terminal part of the vertebrate *rag*1 gene. Therefore, the transposon this gene is associated with is a N-RAG-TP. It unites the transib and N-RAG-TP elements. It may be an ancient recombinant from which all *rag*1 genes derived. These elements might have traveled a long way from prokaryota to primitive and very complex eukaryota phyla. While the sea urchin RAG1,2 fail to work in the human V(D)J system, its RAG2 binds histone tails as the vertebrate RAG2 does. The distance shortens between the shark and human RAG1: the shark and human RAG1 aa identity is 65% and their similarity is 77% [443, 442].

Immunoglobulin M appears first in jawed cartilaginous fish. B cell receptors and immunoglobulin light (L) chain molecules were synthesized first in cartilaginous fish (chondrichthyes, gnathostomata) [444]. IgG developed from IgY that appeared first in amphibians (frogs). IgA appeared first in reptiles. The original Ig gene cluster in cartilaginous fish includes the single V, D, J and C genes. The heavy (H) chain class switch gene appeared first in amphibians and preserved its basic structure up to its mammalian gene. The αβ T cell receptors (TCR) recognize the peptides presented to them in class I or II major histocompatibility complex (MHC) molecules with restrictions. The β loci contain the D and J segments. The γδ T cell receptors are encoded from the γ gene for the J segments and from the δ gene for the two D segments. This T cell receptor interacts with free antigens without restriction. BCRs, TCRs and MHC appear first in cartilaginous fish. Some innate natural killer (NK) cell receptors are connected with some MHC genes and this connection is preserved up to mammals [445]. The whole genome duplications one and two of the vertebrate genomes occurred in the common ancestor of all vertebrates after the appearance of urochordates and before the out-branching radiations of the jawed vertebrates [446]. The amphioxus (*Branchiostoma floridae*) contains only one MHC-like region and no class I and II genes (the proto-MHC), whereas from the cartilaginous fish upward to mammals, MHCs are represented by large clusters of gene families [447, 448]. The predecessor NK cell receptor and leukocyte receptor genes were already represented in the proto-MHC. Endogenous intronic retroviruses persist within the gene clusters of human MHC class II, as remnants of prior proviral DNA insertions. In multigenic regions (as in MHC gene clusters) these retroviral DNA insertions promote the generation of diversity [449].

The evolution of the immune system in deuterostomes extends from echinoderms (sea urchins) through hemichordates (acorn worms), cephalochordates (amphioxus) and urochordates (sea squirts) to cyclostomata (hagfish and lamprey), where it culminates. Then, from the placoderms to cartilaginous fish (sharks), bony fish, amphibians, reptiles, the extinct dinosaurs, birds and mammals, not in a straight line, but in several divergences, another second adaptive immune system emerges. The first adaptive immune system culminated its course in cyclostomata fish (agnatha), but without B and T lymphocytes, no immunoglobulins and without antigen presentation in the grooves of MHC molecules. If genetically re-arranged antigen receptors are the first signs of adaptive immunity, these appear firmly installed in cyclostomata. If the sea urchin coelomocytes posses such a receptor (*vide supra*), then echinodermata exhibited the first sign of direction of development from innate toward adaptive immunity. *Echinodermata* appear to have possessed in their germ line the *rag*2 element to which through horizontal gene insertion the *rag*1 element joined. The donor of the *rag*1 element might have been a prokaryota, and its vector a transposon [450, 451]. Indeed, the inverted RSS repeats are like those of a transposon. From sea urchins to sharks, from fish to mammals, the span of evolution is

estimated to be close to 900 million years. The span from first cartilaginous fish to mammals is placed at approximately 500 million years. If the distance in time is some 3 billion years from prokaryotes to mammals, then the *rag*1 gene is a champion biological space traveler.

2.5.2 An Ancient Herpesvirus

The end product of the long evolutionary line of HHV-4, the Epstein-Barr virus (EBV), is an inducer of autoimmunity (suspect in systemic lupus erythematosus, SLE; in myelolytic encephalopathies represented by multiple sclerosis), and in lymphomagenesis (suspect in Reed-Sternberg cells of Hodgkin's disease; African Burkitt's lymphoma; B-lineage brain lymphomas in patients with acquired immunodeficiency syndrome, AIDS; NK cell lymphomas of the facial sinuses and mediastinum; in body cavity lymphomas with effusions). As to solid tumors, EBV is active in Chinese lymphoepitheliomas, co-infects with human papillomaviruses nasopharyngeal squamous cell carcinomas, detectable in Japanese stomach adenocarcinomas, and probably co-pathogenic in childhood leiomyosarcomas. In many pathological entities (SLE, multiple sclerosis, malignant lymphomas), EBV co-exists with reactivated latent endogenous retroviruses, or with the pathogenic retrolentivirus, human immunodeficiency virus-1 (HIV-1). Association of EBV with these pathological entities has been repeatedly reviewed, biochemically documented [452, 453] and illustrated [213]. One of the most significant biochemical documentations consists of the interactions of EBV gene product proteins BZLF-1 with crucial pro- and anti-apoptotic cellular elements, p53 and NFκB (and the common, but by now diverged evolutionary lineage of p53 and NFκB is also well documented) [454–458].

Recombinases of diverse derivation are Mg^{2+}-dependent enzymes expressing a magnesium ion-binding site, DDE (D, aspartic acid; E glutamic acid). The enzymes with DDE sites are the transposases, retroviral integrases, innate antiviral-response enzymes RNase H and RNA-induced silencing complexes (RISC), and the RAG recombinases. It was pointed out that paradoxically the pathogenesis of HIV-1 depends on its DDE enzymes (which can be inhibited therapeutically), whereas mutations or inhibitions of the *rag* gene-product proteins, RAG, result is immunodeficiencies and severe illnesses (Omenn syndrome) [459].

Dreyfus found similarities of the structures and functions of the DDE proteins and the EBV DNA-binding protein (DBP), the product of the BALF-2 gene. Thus EBV BALF-2 gene product proteins could interact with the V(D)J recombination process [460] The presence of a *rag*-1-like sequence in EBV genome may be the remnant of an ancient acquisition. A scenario can be envisioned in which the ancestor deuterostomes at an early stage of the line that led to sea urchins, acquired a *rag*1-like sequence from prokaryotes and carried that gene in their germ lines inserted next to their genuine eukaryotic *rag*2 gene. That RAG1/RAG2 complex did not interact with the V(D)J gene cluster, which is non-existent in the echinodermata genomes. As sea urchins and placoderms coexisted in the post-cambrian sea, a herpesvirus infecting the echinoderm sea urchins might have excised the *rag*1 gene from its host's genome and incorporated it into its own viral genome. Host gene acquisition by herpes (and other) viruses has been and remains a common practice referred to as gene drain, usurping and expropriating host cell genes. The deuterostome lines leading to echinoderms (sea urchins) and sharks (placoderms, carcharine sharks; gnathostomata chondrichthyes) diverged into these two directions hundreds of million years earlier, but preserved the germline *rag*2 gene. The herpesvirus, a probable ancestor of extant EBV and carrier of the *rag*1 gene, infected the placoderms and/or carcharine sharks and inserted into their germ line the *rag*1 sequences. There the *rag*1*rag*2 genes encoded their gene product proteins the RAG1/RAG2 recombinase-mediating enzymes interacting with the V(D)J and RSS complexes. Were the V(D)J RSS complexes arriving into the same hosts from other sources (as transposons)? The G/C content of the termini (terminal repeats, TR) of the EBV genome is enriched up to 70%, like the V(D)J RSS A/T rich nonamer region. One of the EBV TR nonamer sequences

is adjacent 5′ to a sequence with V(D)J RSS similarity. In this aspect EBV termini resemble transposon termini. The intracellular herpesviral genomes are either circular in latent episomal position, or replicative linear form inserted in the host cell's genome. The transition of the herpesviral genome from circular to linear form is initiated by transcription factor BZLF-1 gene product protein. In EBV's replication cycle sequences resembling V(D)J RSS are produced [460].

Adjacent to EBV's DNA-binding protein, the product of EBV gene BALF-2, lie response elements AP-1 and SP-1 (activating protein-1; specificity protein). AP-1 is involved in cell proliferation, differentiation and migration and it interacts with the *jun* proto-oncogene (*vide supra*) [461]. The *jun* proto-oncogene disallows cell survival in the autophagic state under distress; it induces apoptotic death of autophagic cells [462]. The SP transcription factors regulate those genes that encode neoangiogenesis factors, and invasiveness of malignantly transformed cells. When the SP factors active in tumor cells (ovarian carcinoma; pancreatic carcinoma) are targeted with antibiotics (mithramycin and its derivatives) or with chemotherapeuticals, overexpressed SP-1 becomes downregulated with cessation of tumor growth resulting [463, 464]. Both AP-1 and SP-1 interact with the RAG proteins, probably indirectly by elevating cyclic AMP. The binding sites of the EBV BZLF-1 protein are similar to those of AP1; these binding sites are located within 2 kb of the BALF-2 ORF. EBV infection of T lymphocytes results in a robust stimulation of RAG activity. The structures of the RAG1 and EBV's DBP show similarity in their N-terminal regulatory domain and in their C-terminal DNA-binding domain. Both proteins display Mg^{2+}-dependent DDE residues (*vide supra*). Both proteins express a zink finger in similar regions. This suggests that these two proteins descended from a common ancestor proto-RAG recombinase, imitating a transib insertion (but for proof more primary sequence similarity would be required). Dreyfus concludes that insertions of DDE recombinase of ancient herpesviral origin occurred adjacent to primordial *rag*2 genes in ancestors of sea urchins or in ancient sharks and the remnants of this RAG1 protein are still expressed in the BALF-2 protein and its promoter in extant EBV particles [460].

What could be the value of *rag* sequences in the genome for a herpesvirus? Since structural and functional similarity is evident between the RAG and DDE/RNase H family nucleases and the dimethyl arginine argonaute protein component of RNA-induced silencing complex (RISC), such sequences may serve within the defensive mechanisms of the virus within its host cell. Both RAG1 and RISC enzymes utilize Mg^{2+} ions at their DDE site, as another herpesviral DNA-binding protein, infected cell protein-8 (ICP-8). Argonaute and RISC are RNA-silencing elements; argonaute was operational in archaea (*Thermus thermophilus*). The argonaute gene family members underwent extensive horizontal transfers from *Aquifex aeolicus* through archaea and eukaryota [465]. The targets of RISC and argonaute are double-stranded mRNAs. Both EBV and HHV-8 (Kaposi sarcoma-associated herpesvirus) produce large numbers of micro-RNAs (miRNA). The targets of viral miRNAs and interfering RNAs (iRNA; RNAi) are the ds mRNAs translating host cell proteins that are encoded for anti-viral defense. Viral miRNAs form complexes with RISC and argonaute in order to effectively attack targeted ds mRNAs. The small regulatory RNAs (sRNA) are small interfering siRNAs, miRNAs, and piRNAs (pi, piwi, P-element-induced wimpy testis discovered in infertile males of drosophila and mouse colonies). PIWI-RNAs protect the germline genome by eliminating alien retrotransposons of invasive intent [466–471].

Dreyfus writes: "Obviously, it will not be possible to revisit the origins of the acquired immune system, except through empirically testable hypotheses" [458]. Maybe it is possible to simulate the ancient events. Can an experiment be designed in which a herpesvirus devoid of the *rag*1 sequence infects sea urchin caelomocytes to see if it can pick up the sea urchin *rag*1 sequence? If the herpesvirus becomes a carrier of the *rag*1 sequence and it infects deuterostome-derived cells, preferably cells from a primitive cartilaginous fish in lack of *rag*1 sequences (but carrying germline *rag*2 sequences), can the herpesvirus insert its *rag*1 sequence into the genome of the fish cell, and if so, next to the germline *rag*2 sequence [213]?

In its May 1, 2009 issue volume 324, pages 580–581 *Science* published a science writer's assessment "On the origin of the immune system." Of the letters the editors received in reply to this article, they published only one [472], and well deservedly so. It was D. H. Dreyfus' letter entitled "Immune system: success owed to a virus?" One of the other letters submitted to *Science*, but not published, is printed here[3].

2.6 Horizontal Gene Gains in Eukaryota

2.6.1 Viral Genes

2.6.1.1 Bornavirus in Human Brain Cells

In Borna, Germany, a strange neurological disease ("die Bornasche Krankheit im Bereich Borna/Leipzig, der Kreisstadt Borna in Sachsen, die bereits seit über 200 Jahren bekannt") of horses was observed over 200 years ago ("Gehirn und Rückenmarkentzündung der Bornaschen Krankheit beim Pferd"). In 1885 in Saxony, Germany, next to the township Borna, the German cavalry lost many horses to a disease of unknown causation ("als Verursacher einer tödlichen Hirnerkrankung bei Pferden"). Intranuclear eosinophilic Joest-Degen inclusion bodies in brain cells of horses afflicted with the disease [473], lymphocytic infiltrates of involved brain tissue, and transferability of the disease with cell free extracts to rabbits, rats and mice strongly suggested a viral causative agent for Borna disease. The causative agent was replicated in tissue cultures including human cells. The Borna disease virus (BDV) was identified as a ssRNA virus consisting of 8,910 bp; it is a unique member of the *Mononegavirales* group, which possess an RNA genome with a sequence opposite to a mRNA. The viral nucleoprotein gene encodes a viral structural protein for the packaging of the viral RNA genome in the nucleocapsid. BDV is not explicitly cytopathic as infected cell may survive, but the immune reaction thus elicited may kill infected cells ("BDV verursacht die Bornasche Krankheit, eine virus-induzierte, immunvermittelte Entzündungsreaktion des zentralen Nervensystems"). In addition to horses, the pathogenicity of BDV is quite broad, both by experimental transfer of the disease to rabbits, rats, mice (rat brain virus transferred by intracerebral injection into newborn mice) [474], tree shrews, cats, rhesus monkeys, and by its natural infections through unknown routes to sheep, birds

[3]THE FABULOUS TOPIC OF JOHN TRAVIS' ESSAY continues to excite biologists, geneticists, immunologists, retro- and herpesviral virologists and even oncologists. It was in the belly of the chondrichthyes gnathostomata sharks, where the elements of the adaptive immune system, previously existing in primordial forms dispersed in the amphioxus, ascidian tunicates (the Botryllus), mollusks, anemones and sea urchins, united to work together for the first time. The system withstood the challenge of placentation in mammals, but fails to promptly control epidemics. The malignant cell masquerading as "self" manages to recruit both the innate and the adaptive immune faculties of its host for its own advantage. The innate natural killer (NK), and the adaptive immune T cells can recognize and kill cancer cells. However, the subverted cancer-bearing host mobilizes $CD4^+CD25^+FoxP3^+$ regulatory T cells and induces an armada of innate chemokines to efficiently antagonize NK and immune T cells within the tumor. In a desperate effort, clones of interferon-γ-producer $ICOS^{hi}$-expressor (inducible costimulator) $CD4^+$ T cells rise under the effect of CTLA4 blockade (cytotoxic lymphocyte antigen) to react to cancer antigens and to outnumber the regulatory T cells. Another overstimulated TGFβ-producer and $ILEI^+$ (interleukin-like ETM-inducer) $CD8^+$ T cell clones emerge to initiate the process of ETM (epithelial to mesenchymal transition) in parenchymal stem cells, and in enlisting the ras proto-oncogenes, promote the malignant transformation of these misled stem cells. These T cells commit high treason against their host. This author proposes that these clones be referred to as those of traitor/transforming T (T/T T) cells. However, ras-transformed tumor cells can not produce interferons. Thus, these transformed stem cells succumb to infection with oncolytic viruses. The purpose of this brief note is to direct the attention to a recent lavishly illustrated and referenced monograph and to articles elaborating on these issues in great detail. The title of the last article is "Horizontal gene transfers and cell fusions in microbiology, immunology and oncology." Joseph Sinkovics.

(from psittacine birds to ostriches) and to human patients ("vom Tier auf den Menschen übertragbare Erkrankung"). The first proposal that BDV infects human beings and that it causes neuro-psychiatric disorders was entirely unacceptable to learned retrovirologists of the highest rank. The laboratory documents that anti-BDV antibodies and antibody-virus antigen immune complexes have been identified in the blood of patients with neuro-psychiatric disorders ("dass Menschen Antikörper haben können") were rejected as experimentally erroneous and thus unacceptable (unconfirmable). The authors of such publications were silenced (ordered to refrain from such publications and lectures) by executive orders from the institute director. However, the laboratory documentation of antibody production in human patients with neuro-psychiatric disorders was repeatedly confirmed by independent investigators and on occasions BDV isolates from human patients were reported. The German Institute of Laboratory Medicine held a conference in Berlin in January 2008 on BDV infections in animals and in human patients and published the material of the entire conference in the Acta Pathologica Microbiologica et Immunologica Scandinavica in 2008 [473]. In this volume, B. Norrild, the author of the Introduction, and H. Ludwig, the author of the Epilog refer to the authoritativeness of the institute director declaring that such dictatorial orders are peremptory to the freedom of medical research (cited in [473]). The author (L. Bode), who was ordered to refrain from presenting data from her laboratory and clinics concerning the human pathogenicity of BDV, spoke at the congress under the protection of a disclaimer: "this article reflects the author's but not the institutions opinion" (cited in [473]). This valuable volume discusses the biology of bornavirus (H. Ludwig, pp. 14–20), the neuropathology and pathogenesis of bornavirus diseases (G. Gosztonyi, pp. 53–57); other authors' presentations concern the worldwide distribution of bornavirus diseases from Scandinavia to Australia and Japan, and very extensively the human pathogenicity of the disease in children and adults (cited in [473]).

Even in the era when inadequate laboratory tests could not equivocally prove the validity of "Koch's postulates" as to the etiological role of BDV in human neuro-psychological diseases, the freedom of publication of new ideas prevailed in several editorial offices [475–485].

The classical publications on persistent infection by Borna virus in the central nervous system, including the human brain, are those of Bode, Gosztonyi and Ludwig [484, 486–488]. Borna virus-exposure, as documented by antiviral antibodies is the serum, and its connection with psychiatric (cyclic affective) disorders was first claimed by Rott et al. in 1985 [475].

It has now been firmly established that the non-segmented, negative sense ssRNA BDV establishes a persistent infection in brain cells preferring for its host cells those residing in the limbic systems of the brain. In mammalian brain cell nuclei even without active viral replication, sequences of the viral nucleoprotein (N) gene may persist. These are endogenous borna-like N elements (EBLN) inscribed in the germ line genome of the cells. Some EBLN elements display ORFs and their mRNAs; thus, the sequences may encode proteins of unknown physiological function in their hosts. The human EBLNs express ORFs suggestive of protein encoding capacity. Could this activity be psychogenic-pathopsychogenic in the human brain? Several ancient insertional events for EBLNs can be detected in the genomes of mammalian cells beginning in primates, dating back to pre-human hosts (40 million years ago), and in squirrels with more recent (10 million years ago) acquisition. Some EBLNs lost sequences and exist as inert pseudogenes. The in vivo events of natural insertion of EBLN elements into the genome of brain cell nuclei could be duplicated in vitro in tissue cultures of human cells infected with BDV [489]. These authors state: "Our results provide the first evidence for endogenization of non-retroviral virus-derived elements in mammalian genomes. . ." [489]. However, the genome of the exogenous negative sense ssRNA virus, the lymphocytic choriomeningitis virus of the Arenavirus class, performed an illegitimate recombination event with the genome of the endogenous intracisternal A-type retrotransposon, which reverse-transcribed the entire recombined genome of both viruses into a cDNA and integrated it into the host cell's genome [490]. Thus, most anything can happen between viruses co-infecting a host cell. The BDV intragenomic sequences are retropositioned probably by LINEs (long interspersed nucleotide element); LINEs are mobile DNA elements (their RNA retrotranscribed into DNA) that copy themselves and excise and reinsert themselves in

host cell genomes. Promiscuous LINEs can act upon non-self LINE templates [491]. and being especially hyperactive in the human brain [492], possibly can retrotranscribe BDV RNA into a DNA strand [493]. Further, non-retroviral RNA viruses, the dsRNA totiviridae, are able to horizontally transfer and integrate their genomes into fungi (candida, penicillium, uromyces) [494] (*vide infra*).

2.6.1.2 Human Herpesvirus-6 in Human Telomers

The phylogenetic comparison of herpesviral genomes show overlap between alpha (equine HV-1, human HSV-1, 2, varicella-zoster VZV, Aujeszky HV) and beta (human CMV, HHV-6) herpesviruses, while gammaherpesviruses (HHV-4 EBV, marmoset HV saimiri, bovine HV-4) are diverse, but without overlap with the αβ-classes. HHV-6 is an ancient human herpesvirus; its genome is the closest to the human progenitor herpesviruses [495]. HHV-6AB are closely related to human CMV and HHV-7 [496]. The overall nucleotide sequence identity of HHV-6A and HHV-6B is 90%. HHV-6AB, especially HHV-6A, are opportunistic pathogens in immunocompromised patients. While HHV-6B causes exanthem subitum (*Roseolavirus*) in children, HHV-6AB association with Stevens-Johnson's syndrome or with Langerhans cell histiocytosis [497, 498] does not prove etiological role of these viruses in these pathological entities. HHV-6AB act as co-factors in lymphomagenesis (including Hodgkin's disease) and in the etiology of multiple sclerosis. Indeed, HHV-6 co-infects CD4 T lymphocytes and promotes HIV-1 replication in AIDS [499]. A HHV-6 genomic sequence encodes a 490 aa polypeptide REP protein), which is homologous to the human adeno-associated virus type-2 (AAV-2) *rep* (replication) gene product protein. This gene in HHV-6 probably was horizontally acquired in a eukaryotic host cell co-infected by both HHV-6 and AAV-2. In such an association, HHV-6 mediates the replication of the helper virus-dependent parvovirus AAV-2 [500, 501]. A 1,473 bp genomic sequence of HHV-6A encodes the transformation suppressor protein (ts → TS). The TS displays 24% identity and 51% similarity to the Rep protein of AAV-2. Both HHV-6A TS and AAV-2 REP suppressed Harvey-*ras* gene-induced transformation of NIH 3T3 cells, but not when *ras*-mutated Finkel murine osteosarcoma retrovirus induced sarcomagenesis. When tested against HIV-1 LTR promoter, REP and TS inhibited it, but the retrolentiviral transactivator response (TAR) element reversed the inhibition [502].

The first proof for the integration of HHV-6 genomic sequences into the genome of a human cell was provided in Japan by the hematology team of Professor Isao Miyoshi[4]. By FISH technique and PCR, the HHV-6 genome was shown to be integrated in the long arm of chromosome 22 (22q13) of an EBV$^-$ Burkitt's lymphoma cell line. The integrated viral genome could be activated to replicate by phorbol acetate and calcium ionophore [503, 504].

That genomic segments of HHV-6 integrate into human chromosomes other than chromosome 22, the chromosome 17 (17q13.3), was immediately confirmed [505]. Transmission of integrated HHV-6 genomic segments from parent to child by vertical inheritance was reported [506]. The chromosomally integrated and vertically transmitted HHV-6 genome is present in every cell of the body. In a most peculiar way, the HHV-6 genomic segments integrate most frequently within the telomere region of the targeted chromosome. These ribonucleoprotein (reverse transcriptase) holoenzymes maintain the telomere lengths of chromosomes. HHV-6 integration sites were 9q34.3, 10q26.3, 11p15.5, 17p13.3 and 19q13.4. Integration actually within chromosome was best documented for the 9q34.3 site [507].

[4]Resident fellows Dr. Isao Miyoshi and this author served together in 1959 at the Department of Medicine of the University of Texas M. D. Anderson Hospital, Houston, TX [213]. This author had the privilege to personally meet and know Dr. Dennis Burkitt in Kenya and Uganda, Africa, in 1966. He succeeded in visiting with Professor Isao Miyoshi on the occasion of the 9th International Cancer Congress held in Tokyo in 1966.

In sequencing the HHV-6A genomic integration sites in patients with families of integrated and inherited HHV-6, at chromosomal sites 17p13.3, 18q23, and 22q13.3, the telomeric repeats TTAGGG were found to be the integration loci. In chromosomes with integrated HHV-6A genomic sequences, no circular episomal viral genomes were found and the integrated viral genomes could be chemically activated to replicate [508]. Patients harboring integrated HHV-6 genomic segments become immunosuppressed, when latent viral genomes switch to replicative state. HHV-6 not only attacks lymphocytes representing adaptive immunity, it also blocks signaling from TLRs. In HHV-6-infected DCs, LPS-stimulated TLR4 do not generate immune reactivity [509].

Telomerases are regulated by transcription factors acting on their promoters. Herpesviruses express attractions to telomeres. The HHV-8/KSHV evolved in Africa from the rhesus retroperitoneal fibromatosis herpesvirus (RFHV) to be a human pathogen, Both RFHV and KSHV use identical strategies to parasitize their target cells, to induce their proliferation without killing them, and to reduce the expression of their MHC surface antigens to render them invisible to host T cells [510]. In Kaposi sarcoma cells, it is the latency-associated nuclear antigen of the HHV-8 Kaposi sarcoma-associated herpesvirus (KSHV) that transactivates the telomerase promoter [511]. The product protein of ORF12 of HHV-8 is kaposin, the KSHV's transforming "oncogene-oncoprotein," the unique property of HHV-8/KSHV [291]. HHV-8 is not known to integrate into its host cell genome in its tumor, the Kaposi's sarcoma, which is a "breeding ground of herpesviridae." This tumor harbors in lymphocytes infiltrating it HHV-4 (EBV) and HHV-6, and in the tumor cells CMV, and HHV-8 and an activated endogenous retrovirus (Fig. 2.3a,b) [213, 512].

2.6.2 Horizontal Gene Transfers in Eukaryota

The first eukaryotes constructed their genomes by adding to their vertically preserved gene repertoire genes horizontally transferred from cells with which the "last eukaryotic common ancestor" (LECA) fused. The first chimeric eukaryotes built their nucleus, cytoskeleton and locomotion (cilia) from horizontally acquiesced donated genes [513–516]. However, it might not have been acquiescence from the part of LECA in tacitly complying with the invasion of its genome by exogenous genes. It might have been a voracious engulfment of alien genes and genomes for the achievement of superiority over other cells in competition for niche and nutrients. LECA was a "conscious cell" with microtubules that were evolving into neurotubules [517, 518]. Beneath the Darwinian threshold (*vide supra*) widespread distributions and exchanges of archaeal and prokaryotic/eubacterial genes occurred admixed with vertical inheritance. The repeated lateral transfers of the archaeal reverse gyrase into thermophilic bacteria (*Thermotogales; Aquificales*) by plasmids and transposases [519] exemplifies the promiscuous life style of the early proto-spheroplasts. The era of speciation above the Darwinian threshold favored the events of organelle acquisition by endosymbiosis. Excised and re-inserted "genomic islands" carrying virulence and antibiotic resistance gene clusters traveled between related species [520].

The photosynthetic protist, *Euglena gracilis*, acquired its transketolase nuclear genes and other genes from multiple endosymbiotic events, most prominent being the one with cyanobacterial plastids [521–523]. The eukaryotic organelles, plastids, derived from cyanobacteria through endosymbiosis, as protists captured and engulfed green and red algae. The early eukaryota experienced primary, secondary and tertiary endosymbiotic events resulting in the horizontal movements of the plastids. Most of the plastid genes ended up in the nuclei of the host cells [524, 525]. The apicoplasts, the remnants of engulfed red algae, remain either as relics, or as functional chloroplast-like organelles in some apicomlexan unicellular eukaryotic parasites (*Eimeria, Plasmodia, Theileria, Toxoplasma* sp.). Ciliates diverged into dinoflagellates and apicomplexan parasites. Plasmodia at one time possessed both chloroplasts and mitochondria, both much reduced in size and function in the extant host. In *P. falciparum* proteins encoded in the nucleus (endoplasmic reticulum-associated protein degrading

enzymes) communicate with the apicoplast [526–528]. Both plasmodia and toxoplasma express special nuclear genes (*pfprex*) to encode proteins (DNA helicase, polymerase and primase) with affinity to the apicoplast. In response, the apicoplast contributes encoded proteins to host cell metabolism (the pyruvate dehydrogenase complex). The plasmodium apicoplast gene *tuf*A encodes a translation elongation factor (EF-Tu), which, however, renders the red cell-parasite host susceptible to the chemotherapeutic drug thiostrepton [529–531]. The presence of horizontally transferred genes of mitochondrial origin within apicoplasts strongly suggests that these two organelles of the same host cell co-operate in the interest of their host [532].

First, parasitic chlamydiae gained genes of important enzymes from free-living actinobacteria [533–535]. Then, the transferosomes of chlamydiae transferred genes through the routes of "endosymbiotic gene transfer" to their unicellular eukaryotic hosts [536]. This paper gives a spectacular view of the details of this ancient association. Among the laterally acquired gene winners are human parasites (entameba, trichomonas) and the free-living amoeba, *Dictyostelium* [537–539]. Some horizontally transferred genes travel via transposons, "the most abundant, most ubiquitous genes in nature" [540]. Marine invertebrate crustaceans yield some of the most ancient mariner-like elements (transposons); for example the Bytmar1 transposon in the hydrothermal crab *Bythograea* [541]. Some transposons find their way to the nucleus with the help of (attachment to) DNA-binding proteins [542]; some are transferred by viruses or plasmids (by phages in bacteria and by their descendants, all the eukaryotic viruses). Cyanophages (cyanomyovirus related to T4 coliphage) operate with 64 genes. Of these, there is a highly conserved hyperplastic region containing inserted genes of host cell-like sequences encoding enzymes (plastoquinol, plastocyanin, 6-phosphogluconate and glucose 6-phosphate dehydrogenase) [543]. The *Rhodothermus marinus* RM378 phage encodes a polynucleotide kinase with some sequence similarities to the same enzyme of coliphage T4. These enzymes work to counter the anti-phage miRNA defense of the bacterial hosts [544]. The T4 coliphage (phi1) encapsidates small circular host cell DNAs for horizontal transfer to new host cells [545]. Even rickettsiae release plasmids to communicate with their host cell, ranging from those of arthropod vectors to that of mammalian hosts [546].

Genes and gene product proteins now recognized as proto-oncogenes and oncoproteins appeared first to perform physiological functions, that they preserved (reviewed in [213]). The origin of the Ras family of widely multifunctional proteins (Kirsten and Harvey rat sarcoma oncogenes, *ras,* in multicellular eukaryotic hosts) could be traced back to prokaryotes. The prokaryotic MgIA proteins of eubacteria, and one archaea (after *E. coli* methylgalactoside transport operon) appear as analogues of the Ras proteins showing the five characteristic motifs of their guanosine GDP/GTP-binding pattern [547].

Genes not part of the hosts' vertically maintained ancestry are those of prolyl-tRNA and alanyl-tRNA in diplomonads and parabasalia. The origin of these genes could be traced back to the hyperthermophile *Nanoarchaeum equitans.* It is unsettled if there is a common archaeal ancestor for *Nanoarchaea* (diverging from *Crenarchaeota* and *Euryarchaeota*) and the diplomonads and parabasalia, the first independent eukaryota to appear after the divergence of the archaeal lineages [538]. Protochordate ascidian larvae utilize cellulose synthetases (CesA) of prokaryotic origin for the formation of the long cellulose fibrils along the larval tail. In CesA knockout larvae, the notochord cells were misaligned and the tail failed to elongate. The CesA gene was inserted from a prokaryotic donor into the early lineage of the tunicates. Extracellular cellulose microfibrils started the morphogenesis of the notochord and tail in the larvae of *Oikopleura dioica* [548].

The yeast cells *Candida parapsilosis* and *C. tropicalis* use the CTG codon to translate leucine, as if it were serine. These yeast cells might have acquired this anomaly from an ancient proteobacterium. *C. parapsilosis* lost its phenazine superfamily (PhzF) gene cluster, but re-acquired it through horizontal transfer from proteobacteria [549]. Bacterial genes encoding arsenite reductase, catalase, racemases and peptidoglycan metabolism enzymes show up in fungi [550].

Horizontal exchange of genes between plants and fungi occurs in both directions [551]. Fungal pathogens of plants include the basidiomycetes *Moniliophthora perniciosa* attacking cacao plants causing the "witches' broom" and "frosty pod rot" diseases. These fungal species horizontally acquired genes from oomycetes (the necrosis inducing proteins), from actinobacteria (the metallo-dependent hydrolase) and from firmicutes/bacteriodetes (the mannitol phosphate dehydrogenase) rendering them pathogenic [552]. Interspecies horizontal gene transfers render saprophytic fungi pathogenetic [553]. In the genome of *Aspergillus fumigatus* segments containing 214 alien genes were detected. These genes were of bacterial (40%), fungal (25%) and viral (22%) origin [554]. A tobacco plant plastid inserted its DNA into the soil of Acinetobacter sp. The inserted sequence consisted of a leucyl-tRNA encoded by tobacco gene *trnL*, and an anchor sequence of an antibiotic-resistance (to spectinomycin and streptomycin) gene cluster [555]. Angiosperms resist horizontal gene insertions from fungi, but in rice plant (*Oryza sativa*) genomes five fungi-to-plant and 4 plant-to-fungi horizontal gene transfers are documented. Such gene exchanges are very rare (14 in 3,177 gene families examined), but may result in phenotypic changes of the recipients [551]. Plant pathogenic *Fusarium* spp. possess pathogenicity-related chromosomes and by horizontal transfer render non-pathogenic fungal strains pathogenic [556].

Flowering plants may increase their beauty by the acquisition of horizontally transferred mitochondrial genes encoding ribosomal and respiratory proteins [557]. In rice, maize and sorghum, a LTR-retrotransposon (Route66) transfers horizontally genomic sequences [558]. However, from transgenic rice, the trehalose phosphate synthase and phosphatase genes were not (as yet) transferred to soil microorganisms in paddy rice fields [559].

Crown gall disease and hairy root disease are caused by the phytopathogenic bacteria *Agrobacterium tumefaciens* and *A. rhizogenes* [560]. Interkingdom horizontal ssDNA transfers between plants are mediated by agrobacteria. The T-DNA of plant-transforming *A. tumefaciens* travels to the targeted plant cell nucleus via the ssDNA-binding VirE2 protein; the phosphorylated VirE2 interacting protein VIP1 accomplishes the nuclear targeting of the agrobacterial T-DNA [561]. Plasmids and their vectors (*A. tumefaciens*) for expression of heterologous genes in transgenic plants by horizontal transfer are commercially available [562–565]. The alpha- and beta-proteobacteria, *Rhizobia*, form nodules on the roots of legumes, within which they enter plant cells to fix atmospheric nitrogen. Symbiotic plasmids of rhizobia horizontally transferred to the pathogenic *Ralstonia solanacearum* converted its pathogenicity to mutualism (symbiosis). It was the inactivation of the *hrc*V structural gene that allowed nodulation and the inactivation of the *hrp*G master virulence regulator gene product protein (HrpG) and hypersensitive response and pathogenicity *(hrc* = *hrp* conserved). This process allowed the intracellular entry of the bacteria, which then accomplished nitrogen fixation, thus modulating the transformation from pathogenicity to symbiosis [566]. Extensive horizontal exchange of genetic material occurs between plant cells in the process of grafting [567].

The plant-parasitic "root-knot nematode" *Meloidogyne* sp. acquired genes (L-threonine aldolase; glutamine synthetase; N-acetyltransferase) from sympatric rhizobia, *A. tumefaciens* (also called *Rhizobium radiobacter*) and *R. leguminosarum* [568].

Some diplomonad protists do not have mitochondria, but very likely, after transferring mitochondrial genes from a symbiont proteobacterium, got rid of the original structure. Two anaerobic intestinal parasites, one in the salmon (*Spironucleus salmonicida),* one in the human gut (*Giardia lamblia, G. intestinalis, G. duodenalis*) diligently collected (84 of them) horizontally transferred genes. Most of the horizontally transferred genes originated from prokaryotes, but some of them were recognized as of eukaryotic derivation. A glucose-6-phosphate isomerase (G6PI) gene was donated by the cyanobacterium Nostoc [569, 570]. The common ancestor of these diplomonads was aerobic; the acquired genes helped the development of anaerobiosis in the intestinal tracts. Cystein-rich surface proteins serve as virulence factors; nineteen lineage-specific gene acquisitions distinguish the two lines of the diplomonads, as to their host selection [539].

Bacteria acquired alpha2-macroglobulins for colonization factors by horizontal transfers from metazoa. The *yfh*M/*pbpCV* tightly linked genes (named in *E. coli*) encode α2-macroglobulins and peptidoglycan transglycosylase (for abbreviations of *yfh*M/P76578 and *yfa*S/P76464, and further explanations as to donor metazoan: toby.gibson@embl.de [571].

The non-coding RNA-1 for heat shock response (HSR1), the stimulation of heat shock factor-1 (HSF1) in eukaryota (mammalians) derives from bacteria. The mammalian HSR1 consists of 604 nt; there is only 4 nt difference between hamster and human HSR1. However, the amino terminal regions of the bacterial chloride channel proteins (in *Burkholderiales*) and the ORF of HSR1 are close to identical. This suggests the horizontal acquisition of the original eukaryotic HSR1 sequence [572].

The sponge species *Reniera*, the sea anemone species *Nematostella*, the amoeba species *Dictyostelium discoides* possess very similar α-amylases. Does this mean horizontal gene transfers between these eukarya? [573].

The small spore-forming obligately intracellular pathogenic eukaryota microsporidium, *Encephalitozoon cuniculi,* lacks protein kinases (MAP kinase cascades, AMP-activated protein kinase, stress-response, ion homeostasis, nutrient signaling protein kinases) in comparison to those in *Saccharomyces* yeasts. *E. cuniculi* and the yeasts shared a common ancestor that lived 800 million years ago. The *E. cuniculi* genome lost its old ancestral yeast meiosis kinases, but retained core cell machinery kinases (Aurora, Polo, etc); its kinome consists of only 32 protein kinases [574].

E. cuniculi depends on its host cell's metabolism, yet it is not a symbiont, but a lethal pathogen. Tandem repeat DNA regions in different isolates of *E. cuniculi* indicate that sex by recombination occurs between *E. cuniculi* individuals. Further, *E. cuniculi* managed to receive genes by horizontal transfer from co-intracellular resident Chlamydia. The zygomycete *Rhizopus oryzae* shares germ line genes with *E. cuniculi*. These two microsporidia might have shared a common ancestor [575].

The eukaryotic phylum, Apicomplexa, includes among others plasmodia, toxoplasma, and cryptosporidia (*vide supra*). The intracellular endosymbiont and pathogen, *C. parvum,* is the recipient of horizontally transferred genes. Deprived of its plastid (apicoplast), it depends on genes coming from other apicomplexan parasites, or from bacteria. The gene for leucine aminopeptidase from cyanobacteria is present in the genomes of cryptosporidia, plasmodia and toxoplasma. Other enzyme-encoding genes derive from proteobacteria. Description of the mode of acquisition does not mention viral transfer. However, algal or cyanobacterial endosymbionts of cryptosporidia are possible sources. There is no explanation for a leucine aminopeptidase plant-like gene in the cryptosporidium (and plasmodium). The "plethora of prokaryotic genes" might have derived from the mitochondria, but their acquisition time is much more recent, than that of the acquisition of mitochondria [569]. The *Entamoeba histolytica* genome encodes an endonuclease that was transferred from a bacterial source (EhLINE1) [576]. In reverse, the intra-amoebal *Legionella drancourtii (vide supra)* acquired its sterol reductase gene from its eukaryotic host [577]. Prokaryotic enzyme-coding gene donations show up in *E. histolytica* and *Trichomonas vaginalis* [578].

The sea slug, *Elysia chlorotica,* feeds on the alga *Vaucheria litorea*. The algal cells are digested, but the algal plastid (chloroplast) remains intact and continues its photosynthetic activity in the sea slug. There may be a virus involved in the incident by transferring to the sea slug the algal gene(s) that encode(s) photosystem complex substances needed to keep the chloroplast functional [579].

Prominent intracellular bacteria are *Anaplasma, Ehrlichia* and *Wolbachia.* Wolbachia are alpha proteobacteria. Wolbachia A infects drosophila, WO-B infects insects other than drosophila, including mosquitos, and WO-D infects nematodes. The horizontal transfers range from less than 500 bp to the entire wolbachia genome (>1 Mb) and involve 4 insect and 4 nematode species as recipients [580]. The α-proteobacterium, in the order of Rickettsiales, *Wolbachia pipientis*, interferes with reproduction, kills male insects, or feminizes them. Wolbachia genomes express up to 1,386 coding sequences [581]. The one-way crossing incompatibility between infected males and uninfected females is the event of "cytoplasmic incompatibility". The male testicles are infected, but the sperm cells do not carry live wolbachia; female egg cells are infected with wolbachia. The infected paternal

chromosomes are lost, while the uninfected female chromosomes segregate properly yielding haploid male progeny. When an infected male fertilizes an infected egg cell, and the infecting wolbachia strains are identical, embryonic development proceeds unimpeded. Some Wolbachia sp. infects filarial nematodes. The wolbachia genes transferred into the insect beetle several million years ago, were found now to be disrupted, rendered transcriptionally inactive and turned into pseudogenes [582]. At least some of the wolbachia genes transferred into the mosquito *Aedes* sp. remained functional after an extended period of their horizontal transfer. In mosquitoes, the transferred wolbachia genes encoded receptors for malaria plasmodia in the insects' salivary glands. These wolbachia genes appear to have been originally acquired by the wolbachia genome from another insect host through lateral (horizontal) transfer [583]. The mosquito gene AAE-L004181 shows 50% aa identity with two wPip genes WP1348 and WP1346. Which one is the gene donor and to whom? Wolbachia-to-host transfer was proven [584]. The outer membrane proteins of wolbachia cells are inducers of innate immune reactions in insects, and are also apoptosis-inducers [585]. In drosophila sp., horizontally acquired transposons/retrotransposons abound, but not of wolbachia derivation. Insertins of LTR retrotransposons are the most frequent (90%), whereas non-LTR retroelements seldom (6%) succeed at their self-insertion [586, 587]. Almost the entire wolbachia genome is inserted in the bacteriome of the bedbug (*Cimex lectularius*). The wolbachia genome is vertically transmitted within the oocytes; bugs deprived of the wolbachia genome become infertile [588].

The wolbachia genomes inserted into the genome of insect hosts carry their prophages. Wolbachia phage WO-A is a pyocyaneus-like element. Wolbachia phage WO-B matures into particles, which spread horizontally between different strains of wolbachia within their insect hosts. The WO-B phage expresses genes that influence the biology of the insect host (virulence function gene; sex-specific expression genes). Wolbachia genomes are in the insects' sperm cysts and in the egg cells. The bacteriophage WO-B in the maternally inherited wolbachia genome is most active (replicative, temperate) in the larval stage of the insect hosts. Lytic phage activity may kill (lyse) the endoparasitic wolbachia [584, 589–592]. Whatever happened between wolbachia and the arthropod hosts millions of years ago, it is the scenery of a past mortal combat between host and parasite what is replayed today (as the Hubble telescope shows what happened in the universe milliards of years ago).

In another relationship, the arthropod Antarctic springtail (*Cryptopygus antarcticus*) acquired from bacteria its endo-β-1,3-glucanase gene [593]. The parasite of legume hosts, the pea aphid (*Acyrthosiphon pisum*) appears to have acquired a functional gene from a wolbachia; the gene product protein serves the aphid's endosymbiont, the *Buchnera aphidicola* [594]. The red-green colored carotenoids are encoded in pea aphids by genes of fungal derivation; after integration of these alien genes, the aphid genome duplicated them [595]. The 464 Mb genome of the pea aphid *A. pisum* shows extensive gene duplications, gene losses and new gene acquisitions. The expanded genes are set for chromatin modification, miRNA synthesis and sugar transport. Lost genes are those of the urea cycle and purin salvage and selenoprotein utilization, and some of the innate immune system, may be to be able to accommodate the bacterial endosymbiont *Buchnera aphidicola* (a gamma proteobacterium), which the aphid maternally transmits to its progeny. With this endosymbiont, the aphid shares aa synthetic purine metabolic pathways. The aphid possesses the aa synthetic and degrading genes and shares the products with the endosymbiont. In turn, the endosymbiont provides the purin metabolic pathway (purine nucleoside phosphorylase, adenosine desaminase: adenosine to inosine) for the salvage of purine nucleotides, except for guanosine, which is rendered by the aphid [596–598]. At least 12 genes in the aphid's genome are of bacterial origin (named in the article), but most of these genes are not of Buchnera, but are of wolbachia origin [594]. Buchnera and Hamiltonella are facultative symbionts to aphids; this type of endosymbiosis protects the aphids against pathogenic fungi, parasitoid wasps (*vide infra*) and heat strokes [599].

Female wasps use ichnoviruses to immunosuppress the caterpillars so, that in the caterpillars' body the wasp eggs may hatch and their larvae may develop. The caterpillars' immune reaction to the

injected foreign bodies (eggs) would consist of activated Toll-like receptors and phenolooxidase production. The ichnoviruses derive from ascoviruses (polyDNAviruses); descendants of iridoviruses. Ascoviruses injected into caterpillars would kill these hosts, but the ichnoviruses are attenuated and non-pathogenic. Ichnoviruses are restricted to replicate only in the female wasp's calyx cells. Ichnoviral genomes exist in the form of multiple circular DNA molecules. Ichnoviral particles assemble in the female wasps' genital tract. In the caterpillar host, the ichnoviruses do not replicate. The ichnoviral genes are not for encoding new viral particle structural proteins; the ichnoviral genome in the caterpillar encodes a number of proteins, which derive from wasp's genes inserted in the ichnoviral genome. In the female wasp's genome, most ascoviral genes were eliminated from the ichnoviral genome. The only one strain of ascovirus, which is able to replicate both in the wasp and in the caterpillar hosts is the DpAV4 (from *Diadromus pulchellus*). The DpAV4 is more of a symbiotic, than of a pathogenic virus. Symbiotic virus-host relationships promote reciprocal passive lateral transfers of genes between viral and host cell genomes. The viral genome undergoes recombination-primed replication in an environment, where an abundance of DNA sequences float freely. In the amoeba, the large dsDNA viruses (NCLDV, *vide supra*) chose this mode of genome replication. There, bacterial genomes are released from the microorganisms, which amoebae and their unicellular symbiotic algae feed on. The recombination-primed genomic replication promotes the integration of very short DNA segments (12 bp) with sequence homology to the amoebic or viral genomes. In these intracellular environments, passive lateral transfers of DNA segments to and from viral, host cell and bacterial chromosomes readily occur. In contrast, active lateral gene transfers show elements of dedication to conclude the selected process.

Wasps' genomes carry integrated sequences of ascoviral and ichnoviral genomes. In the female wasp's genome, the ascovirus loses its virulence genes and acquires somatic genes from its host. The non-replicating a-virulent ichnovirus encodes the wasp's proteins within its new host, the caterpillar. The wasp's proteins suppress the innate immune reactions of the caterpillar and render this host tolerant toward wasp proteins. The exact mechanisms of this symbiogenesis are not known. Endosymbiogenesis repeatedly occurred in the earliest stages of evolution, when proteobacteria became mitochondria and cyanobacteria became chloroplasts in the cells that engulfed them. The endosymbionts became domesticated servants (or slaves) of their host cells, as the cell nucleus expropriated the symbionts' genes one by one [600]. Female wasps domesticated the ascovirus: the servant's name is ichnovirus.

In conclusion for a most complex issue. Horizontal gene transfers are not limited to the sub-Darwinian threshold era, only the intensity of the events declines. Neither is the process confined to the transfer of one single gene. Insect genomes readily accommodate full rickettsial size genomes. The eukaryotic microalga, *E. huxleyi* (*vide supra*), harbors a large dsDNA virus, the EhV. The alga donated its entire sphingolipid/ceramide biosynthetic gene apparatus (seven genes) to its viral guest [158]. The biflagellated photoautotrophic protist, *Cyanophora paradoxa*, and *Euglena gracilis* (*vide supra*) acquired their transketolases from endosymbiotic green algae [522]. The cryptic viruses of beets and carrots are close to be identical with fungal partitiviruses. Naturally transmitted between related plants by seeds and pollens, the transmission of the entire genomic packages of these viruses, and whatever additional plant host cell genes the viral genomes might have picked up, to phylogenetically distant hosts occurs by horizontal transfers mediated by fungi [601]. Rice, maize, sorghum cells accept horizontally transferred genes by the 280,000 year old *gag-pol* retrotransposons called "Route66." Route66 was discovered in the genome of Japanese rice plants (*Oryza sativa*) in two copies residing on chromosomes 2 (nt 1 767 933 – 1772 818) and chromosome 6 (nt 26 706265 – 25 701 456) [602]. Pea aphids readily accept bacterial, genes (but not from their symbiont *Buchnera aphidicola,* it is from bacteria other than *Buchnera*) [603]. Three billion years after transgressing the Darwinian threshold: "are lateral gene transfers between prokaryotes and multicellular eukaryotes ongoing and significant?" [603]. Yes, they are, and very much so.

2.7 Epithelial-to-Mesenchymal Transition of Human Cancer Cells

2.7.1 Bacteria Inducing Inflammatory Cancers

2.7.1.1 Helicobacter Pylori: Cancers of the Soil and Seed

Bacteria acquire and transfer their virulence factors (toxins, adhesins, invasins, etc) through transposons, plasmids and bacteriophages (*vide supra*). Bacterial virulence factor genes are clustered in "pathogenicity islands" PI, Pais) (*vide supra*). Bacteria may delete, or amplify, or duplicate their pathogenicity island genes [604]. Gram-negative bacteria harbor distantly related PIs (*E. coli, Salmonella, Shigella, Vibrio cholerae Helicobacter pylori*). The toxin-coregulated type IV pilus (TcpA) may be encoded by cooperating phages CTXφ and VPIφ. The PI contains the phage genome that encodes the toxin-coregulated pilus (TCP). TCP functions as a colonization factor and as the receptor for phage CTXφ [63a,b] (*vide supra*). The toxin-coregulated type IV pilus (TCPA) expresses the coat protein of the VPIφ phage [63a,b, 605]. *Helicobacter pylori* (Hp) strains seldom succumb to phages; probably effectively eliminate them. The first isolation and photographic depiction of a lytic *H. pylori* phage (HP1) was certainly a sensational occasion. It is worthwhile to look up the original publications to view the electron microscopic depiction of the Hp phages [606, 607]. Genetically modified non-replicating phages lyse Hp, yet clinical phage therapy has not materialized [608]. In the mouse, the lytic filamentous phage M13 prevents the colonization of gastric mucosa by Hp [609]. Some stain of Hp release "rolling-circle replicating" highly promiscuous plasmids [610].

This author reviewed the human pathogenicity of *H. pylori* in the context of "the host confronting pathogens attacking lymphoid tissues" [213]. This bacterium accompanied mankind through its evolution, at least in the last stages of it: it was present 5,000 years ago in the stomachs of the ancient Egyptians. In its extraordinary variability, genomic segments of *H. pylori* strains readily recombine and exchange genes through horizontal transfer [611]. Major subpopulations of Hp are those of hpEurope, hpAfrica (subdivided to hpSAfrica and hpWAfrica), and hpEAsia. The subpopulation hpAmerind parasitizes native Americans. The genomes of the Amerindian (Venezuela) Hp and hpEurope were compared and revealed characteristics of the human Hp strains following the migration of the human race out of Africa, the population of Europe by *H. sapiens* (no information on *Homo neanderthalensis*), but the material from excavated graves would be available), and the movements of human populations through Asia and through the Bering passage-way to the Americas. Even human and murine Hp strains could be compared in an evolutionary scale [612]. The core genome of Hp substrains operates with 1,111 genes, but the gene number may increase to 1,531. The *cag* PI gene clusters may be acquired or deleted. Each Hp strain produces by recombinations highly individualized variants in each of their hosts. In cases of multiple subspecies *H. pylori* infections, interspecies horizontal gene transfers occur [613]. There are conserved and mobile regions in the Hp genome. Gene replacements and horizontal gene transfers directed the evolutionary changes within Hp strains [614]. Practically all infected persons harbor their own individual Hp strain [615].

The pathogenicity of *H. pylori* extends from the epithelial cell linings of the stomach to the reactive lymphocytes infiltrating it, as if it were carcinogenesis in the soil (the epithelium) and seed (the lymphocytes). The cytotoxin-associated gene product protein *cagA* → CagA is phosphorylated by a Src kinase. CagA inhibits E-cadherin and β-catenin complex formation resulting in the transfer of β-catenin from the cytoplasm into nucleus. The cells in which these events take place undergo the precancerous process of "intestinal metaplasia", with goblet cell mucin production in the gastric epithelial cells. When *H. pylori* acquires metazoan α2-macroglobulin genes through horizontal gene transfer, it uses the gene product proteins to suppress anti-bacterial immune reactions of the host [616]. Allelic diversity and extreme genetic variability of *H. pylori* creates individually modified substrains of the bacterium in their hosts [615].

In a "chronic inflammation-induced cancer" scenario, cell nuclear DNA suffers oxidative damage [617]. In these cells, K-ras mutations occur (Kirsten rat sarcoma oncogene). In response, there is first a polyclonal lymphoid cell proliferation. Monoclonally proliferating lymphoid cells emerge overexpressing the anti-apoptotic *bcl-2* gene cluster and undergoing the translocation t(14;18)(q32;q21) [618–620]. While Hp eliminates innate immune reactions by killing macrophages (*vide infra*), it disrupts adaptive immune reactions by generating $CD4^+CD25^+FoxP^+$ regulatory T cells for the elimination of immunoreactive immune T cells ($CD8^+$ T cells) [621].

The c-*myc* proto-oncogene is activated in the nucleus of Hp-infected gastric mucosal cells by ERK (extracellular signal-regulated kinase) and the c-Myc proto-oncogene (avian myelogenous leukemia oncoprotein) is phosphorylated in the cytoplasm. The chain reaction continues with the phosphorylation of c-Fos (Finkel mouse osteosarcoma oncoprotein). The c-Jun proto-oncoprotein (*vide supra*) joins in, resulting in the activation of a specific activator protein, AP-1. These proteins form cytoplasmic complexes, for example, the phosphorylated c-Fos/c-Jun complex binds the c-Myc promoter. The c-Fos/c-Jun complex translocates into he nucleus for further gene activations and gene silencing. The end result is polyamine gene activations with the production of ornithine decarboxylase (ODC), an apoptosis-inducer in macrophages [622]. The macrophages to be killed were lured on the site first by monocyte chemoattractant protein production (MCP-1) by the gastric mucosa [623]. When *H. pylori's* CagA protein translocates into lymphocytes, it assumes the role of an oncoprotein (*vide infra*) (Table 2.1).

The Hp *cag* PI by epigenetic mechanisms hypermethylates CpG (cytosine-post-guanine) islands in the genome of gastric mucosal cells resulting in translocation of NFκB from cytoplasm to nucleus for inflammatory reaction gene activation, AP-1 generation, activation of the PI3K (phosphatidyl inositol 3 kinase) pathway and proto-oncogenic, epithelial-to-mesenchymal transformation-inducing Wnt/β-catenin signaling [624, 625]. Pro-oncogenic and anti-oncogenic but not yet clearly identified microRNAs vie for superiorityin the gastric mucosal cells [626].

In the Spanish coastal town Ubrique the rate of Hp infection is high (54%) with Hp-antibody production shown in blood tests; these tests also showed high incidence (81%) of mutated p53 protein and ceruloplasmin levels in the blood of Hp-seropositive individuals. The mortality rate for gastric cancer exceeded twofold (20/100,000) that of communities with low Hp-related parameters [627]. Helicobacter DNA is present in pancreatic cancer tissue and helicobacter bacteria (but not other gastrointestinal bacteria) appear in hepatocellular carcinoma tissue samples [628]. Pyrosequencing and PCR reveal Hp Dna in liver tissue of patients with chronic cholestatic liver diseases and in gallstones [629a,b, 630a,b]. Since the observation of cytoplasmic vacuolization in gastric mucosal cells by Hp toxin to the documentation of oncogene mutations in these cells only 13 years went by [631–633]. In some patients with idiopathic thrombocyopenic purpura (ITP) and Hp antigens in their stool samples, antibodies to the Hp anticytotoxin-associated gene A product protein were found. After Hp eradication, the platelet levels returned to normal [634]. The association of Hp infection with certain types of ITP occurs world-wide [635–638].

The eradication of *H. pylori* is entirely possible with a combined antibiotics regimen (amoxicillin, clarythromycin or azithromycin, and metronidazole given with an omeprazole-like proton pump inhibitor) [639, 640]. Metronidazole- and tetracycline-resistant Hp stains are emerging in Iran [641].

2.7.1.2 Bacteroides Fragilis: Inflammatory Carcinogenesis with or Without It

This author recently reviewed work initiated at Johns Hopkins Hospital in Baltimore, for the Weekly Hungarian Medical Journal (Orvosi Hetilap) concerning the contribution of enterotoxigenic strains of *Bacteroides fragilis* to colon carcinogenesis [642a,b]. A comparison was suggested between the carcinogenic effect of *H. pylori* in the stomach and that of the enterotoxigenic *B. fragilis* in the colon.

Table 2.1 Helicobacter-induced gastric lymphoma and bacteroides enterotoxin-induced colonic adenocarcinoma

Gastric mucosa-associated lymphoid tissue lymphoma (MALT) in *Helicobacter*-infected stomachs	
Clinical course: Even antibiotic therapy refractory low grade MALTs seldom advance into high grade diffuse large B-cell lymphoma (DLBCL)	[730]
Complete genomic sequences of lymphomagenic *H. pylori* strains	[731, 732]
Chromosomal translocation t(11;18)(q21;q21) results in the formation of API2-MALT fusion oncoprotein. Oncoprotein releases cytoplasmic NFκB and activates Bcl10 nuclear gene	
MALT immunophenotype: $CD19^+CD20^+CD21^+CD79a^+CD5^-CD10^-CD23^-$	
Micro miRNA profile: Aberrant DNA methylation of p16/INK4a gene	
Methylated CpG islands:	[733, 734]
Translocations: t(1;14)(p22;q32) BCL10-IGH oncoprotein; t(14;18)(q32;q21) IGH-MALT1 oncoprotein. Oncoproteins activate NFκB, Toll-like receptors TLR2 & TLR6, chemokine receptor CCR2, clusters of differentiation CD69 & Bcl2. In translocation-negative lymphoma: IL-8 production, CD28, CD86 expression, ICOS (inducible T-cell costimulator) activation	[735]
Dysregulated NFκB pathway	[736]
Helicobacter virulence gene/gene-product protein CagA: CagA translocates (is horizontally transferred) into gastric epithelial cells and into B lymphocytes. Recipient cells phosphorylate (activate) CagA protein. CagA activates ERK and Bcl-2/Bcl-XL anti-apoptotic proteins. CagA is an oncoprotein	[737a,b]
Helicobacter-reactive host immune T cells: Are armed with FasL (ligand) and perforin cross-react in an autoimmune fashion with host cell ATPase autoantigens, kill mucosal cells causing atrophy of gastric mucosa. Immune T cells mobilized against MALT lymphoma B cells are defective in FasL and perforin expression and fail to eradicate B lymphoma cells. In a murine model of *Helicobacter*-induced gastric lymphoma, $CD4^+CD25^+FoxP3^+$ Treg cells were attracted into the tumor by chemokines CCL17/CCL22. The regulatory T cells eliminated immune T cells and promoted tumor growth	[738, 739]
Eradication of H. pylori: Antibiotics sensitive. Treatment results in lymphoma remission	[640, 740]
Commensal bacteria (*Bacteroides vulgatus, Fusobacterium varium*)	
Commensal bacteria adhere to, and enter the cytoplasms of colonic epithelial cells. In response, the cells produce IL-6, IL-8, TNF-α, macrophage/monocyte chemoattractant protein-1. The intranuclear NFκB p65 is phosphorylated (activated). These reactions occur in UC cells in vitro and in vivo, as illustrated	[741]
Bacteroides fragilis enterotoxin activates the REL protein (*vide supra*) heterodimer, NFκB. In response, chemokine (CCL2, chemoattractant for monocytes; CXCL1, growth-related oncogene-α (GRO-α); CXCL8/IL-8) gene overexpression induce neutrophil transmigration	[742]
Comments: Not addressed in this article is the strong possibility that bacteroides enterotoxin-stimulated colonic epithelial cells express FasL, with which they kill Fas receptor-positive host immune T cells. It is well documented that FasL-expressing tumor cells attract granulocytic infiltrations. Genetically engineered immune T cells from patients with metastatic colon cancer express bispecific cytotoxicity to $CEA^+/CD3^+$ colon cancer cells	[743–754]

In the cancer hospital M. D. Anderson in Houston, *B. fragilis* infections were severe and frequent [642a,b, 643] and in general continue to carry high morbidity and mortality [644].

The *B. fragilis* enterotoxin cleaves E-cadherin and activates the β-catenin/Wnt cascade. The enterotoxin induces neoangiogenesis and epithelial cell proliferation. The cell proliferation is driven by the STAT and MAPK cascades (signal transducer activation of transcription; mitogen-activated protein kinase). In mice the enterotoxin failed to induce Toll-like receptor or dendritic cell activations. Instead anti-apoptotic factors (NFκB and c-IAP2) are activated. Epithelial cells of the colon produce IL-8, an NFκB activator. The toxin-exposed colonic epithelial cells exude cyclooxygenase (Cox-2) and prostaglandin (PGE2). Reactive lymphoid cell infiltrates appear expressing IL-17 and the receptor for IL-23. Of the T cell factors, TCF-1 promotes, TCF-4 inhibits Wnt proto-oncogene activation [645–649]. Consequential to unopposed Wnt activity is the upregulation, amplification, or even mutation of the BLC9 (B cell lymphoma) and K-ras genes and overexpression of the EGFR in epithelial cells [650–654]. In contrast to *H. pylori, B. fragilis* is a phage-sensitive bacterium [655, 656] and responds to various antibiotic regimens [644].

In bacteria, and very prominently in *Bacteroides* spp., antibiotic-resistance is mediated by efflux pump P-glycoprotein-encoding genes, which are transferable by plasmids [657a,b, 658a,b]. The same mechanism is operational in chemotherapy-resistant cancer cells. This author could not find comparative studies for bacterial and eukaryotic (protozoal; cancer cells of vertebrates) efflux pump genomics and proteomics, but the mechanisms of action of these entities by ATP-binding cassettes, as well as their inhibition by selected compounds (phenothiazides, chlorpromazine, verapamil) are very similar in bacteria and in tumor cells [659–664]. The bacterial flora in the intestinal tract of gypsy moth larvae rapidly acquires and/or endogenously expresses efflux pump proteins for the mediation of multiple antibiotics resistance (but without any exposure to such antibiotics). Insect guts are environmentally rich in antibiotic resistance genes; there is a high potential for dissemination of such genes from host to bacterial symbionts, and in between members of the bacterial flora [665]. Disiloxanes (SILA-409, SILA-421) excelled as efflux pump inhibitors both in bacteria, where they also suppress plasmid-traffic of resistance genes (*vide infra*) and in multidrug resistant cancer cells [666–668]. The multidrug and toxic compound extrusion (MATE) family efflux transporter gene *bex*A was cloned from *Bacteroides thetaiotaomicron.* The BexA protein sequence is homologous to that of *Vibrio parahaemolyticus*. The *bex*A construct transferred cipro- and norfloxacin resistance to *E. coli* [669].

Most of the antibiotic-resistance genes of *Bacteroides* spp. have been recognized [670–674a,b]. The genes (*tet*Q, for tetracycline, *gyr*A for quinolone resistance, *erm*F for erythromycin resistance, *cfi*A for encoding a lactamase/carbopenemase for carbapenem, imipenem and metronidazole resistance, *cep*A for encoding cephaloporinase, *nim*B for nitroimidazole resistance) are transferred by conjugative transposons (CT, integrative and conjugative elements, ICE), plasmids, and phages, pHag1 and pHag2 [658]. The most prominent conjugative transposons for horizontal transfer of tetracycline and erythromycin resistance-encoding genes (*tet*Q, *erm*F) are the closely related CTnERL and CTnDOT integrases, especially the TcrEmrDOT (dot-blot hybridization, dot-plot) [675–681]. The promiscuous bacteroides conjugative transposons, CTnGERM, carrier of the erythromycin-resistance gene *erm*G, picked up the macrolide efflux pump gene *mef*A from *Streptococcus pyogenes* [682].

Vertically transmitted germ line mutations-induced carcinogenesis in the human colon is very well documented and the oncogenes are cloned and characterized. There are deleted tumor (colon cancer) suppressor genes (DCC from chromosome 18, p53 from chromosome 17p, MCC mutated colorectal caner gene from chromosome 5q), amplified, mutated or translocated oncogenes (c-*myc*, K-*ras*), unique mutated colon cancer-inducer gene (adenoma-polyposis coli, APC) and the Lynch syndrome I-inducer genes (named after yeast genes: PMS, postmeiotic segregation; MSH, Mut(mutated) S homolog; MLH, Mut L homolog, missense mutations). These are the hereditary colon cancer syndromes, the adenoma (polyposis) to carcinoma sequences, and the hereditary nonpolyposis colorectal cancers [683–687]. Colorectal cancers termed “sporadic” in stages II and III (without and

with regional lymph node metastases) present with different genetic signatures in the Affimetrix array [688].

Carcinogenic somatic mutations generated in the colonic inflammasomes are not inherited, and as such their inducers may be subject to horizontal acquisitions. The Lancet gave credit to Rudolf Virchow for proposing in 1863 that bacteria and the chronic inflammation they induce (leukoreticular infiltrates: "Phlogose und Thrombose im Gefäßsystem") may induce cancers [689]. The high pathogenicity and persistence of endo- and enterotoxigenic strains of *B fragilis* were recognized in the 1990s [690, 691]. The molecular pathogenesis of the bacteroides enterotoxin creates an environment highly conducive to malignant transformation of the mucosal cells of the colon (*vide supra*) [646–654]. The enterotoxin can re-arrange cellular cytoskeletons [692). It activates proto-oncogenes (c-*myc;* K-*ras* [693–695] and the human relative of drosophila mutated gene "legless," the BCL9 gene/gene-product protein (B cell lymphoma)). This gene-product protein stimulates the proto-oncogenic β-catenin/Wnt cascade, whose inhibitor is the dickkopf protein (DKK), which is often eliminated in the course of malignant transformation. This pathway of colon cancer oncogenesis may be shared between hereditary (APC tumor suppressor gene mutation) and inflammatory colon cancers [696]. It is not entirely clear what role DKK1-4 proteins may play in colon carcinogenesis. DKK-1 may be epigenetically inactivated and down-regulated, thus loosing its inhibitory effects on tumor cell growth [697, 698]. DKK2, 3, 4 may be upregulated; DKK-3, 4 are neo-angiogenic; DKK-4 inhibits host T cell enhancement and promotes tumor cell invasion [699–701]. In contrast, DKK-4 was found not to activate, but to inhibit β-catenin signaling, colon cancer cell cycle progression and growth [702]. Vitamin D3 (1α, 25-dihydroxyvitamin D) activates the gene of the Wnt/β-catenin antagonist DKK-1, thus inducing differentiation in human colon cancer cells; at the same time, the vitamin D compound inactivates the gene of the tumor promoter Wnt/β-catenin signaling pathway [703, 704].

Enterotoxic bacteroides further activates NFκB; induces IL-6, IL-8, IL-10 and TGF-β production; inhibits apoptosis, and induces neo-angiogenesis [646–654, 705]. In the inflamed colon, ectopic activation of cytidine deaminase (AID) by TNF-α *via* NFκB induction and/or by cytokines IL-4 and IL-13 results in p53 mutation [705], thus removing a major obstacle to colon carcinogenesis. Mutations of the p53 gene in colonic mucosal cells in ulcerative colitis (UC) may be contributory to carcinogenesis [706]. Colon cancer cells overproduce cyclooxygenase-2 (COX-2), a tumor cell growth promoter [707].

The causative factors of UC could not as yet be identified. High expression of heat shock protein (HSP47) in cancer cells in that condition is considered to be a unique feature [708]. The expression of RhoGDIα, the inhibitor of Rho-GTPases, disorganizes intestinal epithelial cells and is a known promoter of progression of breast and inflammatory colon cancers (rhomboid proteins; guanine triphosphatase; guanine nucleotide disassociation inhibitor; guanosine diphospho- (D-mannose)) [709, 710]. The gene "Wiskott-Aldrich (WA) syndrome protein and FKBP-like" (WAFL, tacrolimus-FK-binding protein) is overexpressed in the colon in inflamed colonic mucosa in UC; normally, it is a membrane traffic protein, whose role in carcinogenesis is unknown [711a,b]. Prominent role is ascribed to IL-6 and STAT3/SOC3 signaling pathway in UC-related carcinogenesis [712]. The activator of STAT2 signaling is IFN-αβ. STAT2 acted as a promoter of chemically-induced colorectal carcinogenesis; deletion of STAT2 was inhibitory to these processes. Thus, the proinflammatory mediator, STAT2 is now recognized as an uncovered co-carcinogenic secret agent [713].

Extracellular, cell-surface installed and intracellular innate immune faculties (Toll-like receptors, chemokines and cytokines; macrophages, dendritic cells, natural killer cells and Nod-like receptors) participate in colonic inflammatory and carcinogenic processes, either in a promotional, or in an inhibitory manner. Two extremely well referenced articles review the field with the conclusions that Nod-like receptors (nucleotide-binding oligomerization domains) protect against inflammatory carcinogenesis [714] and that innate and adaptive immune reaction cooperate in the intensity of the immune reactions, but divaricate in matters of pro- and anti-tumor reactions [715]. Homozygous

mutations in IL-10 receptor genes increased the propensity in patients to the development of colitis; thus, removal of a tolerogenic cytokine's actions promoted fulminant immune reactions to the intestinal bacterial commensals [716, 717]. These reports have avoided giving reference to carcinogenesis; there, not IL-10-mediated host tolerance, but intense immune reactions are needed, like in the IL-10R-mutated patients, or mice. An excellent tabulation lists innate immune responses, the IL-23/Th17-type pathway and other genes involved in inflammatory reactions of the human intestinal tract (chromosomal locations of the involved genes given). Two most impressive cartoons depict the interactions between the arms of innate and adaptive immune faculties. The article closes with therapeutic recommendations, as to the severe inflammatory reactions, but without. mentioning any favorable or adverse effects of the anti-inflammatory therapeuticals on the incidence of carcinogenesis [718]. In contrast, adaptive immune reactions mediated by $CD4^+$ T lymphocytes are mobilized in UC against dysplastic colonic mucosa, but not against normal mucosa [719].

The anti-inflammatory reactions that may promote or suppress the incidence of inflammatory carcinogenesis are the inhibitors of prostaglandins and cyclooxygenases (celecoxib) [707]. The pro-inflammatory transcription factor NFκB is inhibited by fluoro- and tribromsalans, sunitinib, lestaurtinib, ectinascidin, chromomycin and bortezomib [720]. The nuclear receptor PPAR (peroxisome proliferatory-activated receptor gamma) is an antagonist of NFκB upon its transfer from the cytoplasm to the nucleus in order to activate inflammatory reactions-encoding genes [721]. In mice with PPAR-positive colonic epithelial cells and lymphocytes, oral intake of conjugated linoleic acid (CLA) ameliorated inflammatory bowel disease and inhibited chemical carcinogenesis; CLA was ineffective in PPARγ-null mice. Successfully responding PPAR-positive mice had no macrophage infiltrations in the mesenteric lymph nodes; expressed low levels of TNF-α mRNA, and mobilized increased numbers of regulatory T cells [722]. In inflammatory bowel disease, IL-6 and its solubilized receptor (IL-6R) form complexes; these complexes react with CD130 of mucosal T cells (IL-6 trans-signaling). In the epithelial cells, IL-6 induces STAT3 signaling (*vide supra*). Myeloid cells in the inflamed colonic mucosa also secrete IL-6. Both anti-IL-6 and anti-IL-6R monoclonal antibodies and other IL-6 inhibitors are available for the suppression of IL-6-mediated pro- and anti-inflammatory reactions [723].

As to stem cells in the colonic mucosa, in response to the proper cytokines and growth factors, normal differentiation ensues. Inflammatory cytokines are genotoxic and mutagenic; the stem cells so treated emerge as cancer stem cells. The expression of CD133 is a clue for such a transformation [724–726]. Inflammatory rectal fistulous tracts could be cured by stem cell transplantation (bone marrow transplant from HLA-matched sibling to alemtuzumab- and fludarabine-preconditioned, gut-decolonized patient, whose graft-*versus* host disease was treated with prednisone). Full chimerism was established without GvHD [716]. From Russia comes a favorable report on improved clinical course of patients with UC treated with allogeneic bone marrow mesenchymal stem cells [727]. In Hungary, regeneration of the inflammatory damage in the colonic mucosa by stem cells migrating to the lesions was observed. As yet unidentified lymphocytic aggregates and the migrating stem cells appeared to have cooperated in the process [728]. If the thesis stands, that certain inflammatory cytokines may be genotoxic to stem cells embedded in the colonic mucosa [725], it is then essential to recognize those cytokines and cellular elements (Paneth cells) that sustain the integrity and the healthy homeostasis in the bowel mucosa [724, 729] (Table 2.1).

Inflammatory carcinogenesis was recognized long ago as a unique event in the induction of squamous cell carcinomas in Marjolin ulcers (Jean-Nicolas Marjolin, 1828). The concept now has been extended to prostate and breast carcinomas without an identified inflammation-inducer pathogen. Some colon adenocarcinomas may be generated by the inflammatory cascades induced by the *B. fragilis* enterotoxin. In gastric carcinoma- and lymphomagenesis, the *H. pylori* CagA gene product protein initiates the well defined cascade terminating in lymphoma- and/or adenocarcinoma induction [737a,b]. Cag A (cytotoxin-associated protein/antigen) may induce cell senescence or carcinogenesis in the gastric mucosa. In the carcinogenic pathway, c-Myc induces

microRNAs miR-17 and miR-20a for the suppression of p21 cyclin-dependent kinase leading to epithelial-to-mesenchymal transition [737a,b] (*vide infra*).

2.7.2 The Epithelial-to-Mesenchymal Transformation

Epithelial-to-mesenchymal transformation (EMT) of cancer cells is a complex act in the interest of increased virulence of the malignant pheno-genotype. The Ras oncoprotein activates TGF-β (transforming growth factor) production, which activates the *snail* genomic sequences followed by lymphoid enhancer factor (LEF) activation. The Bcl-3 protein translocates into the nucleus to react with N-cadherin DNA; in the cytoplasm, cyclin D is inactivated. When the WNT-induced signaling protein (WISP) is inhibited, E-cadherin expression is reduced in tumor cells, which gain invasiveness. The Snail and Twist proteins collaborate with *ras* and inactivate p53 and Rb (retinoblastoma) proteins, an anti-apoptotic event: tumor cells are now protected from apoptotic death. The SPARC protein (secreted protein acidic rich in cystein) promotes the nuclear translocation of β-catenin, which further increases the activation of LEF. This is the environment in which a subclass of tumor-promoter T-Lymphocytes is generated. This lymphocyte population induces (either in a causative, or in a coincidental manner) *ras* gene point-mutations in human breast cancer cells. These breast cancer cells metastasize to the regional (axillary) lymph nodes [755]. Sinkovics proposed the term of traitor/transforming T cells (T/T T cells) for this class of host T lymphocytes [756]. The generation of T/T T lymphoid cell clones have nor as yet shown in *B. fragilis* enterotoxin-exposed colon cells, but it is documented in the microenvironment of human breast cancer that a subpopulation of reactive T lymphocytes (T/T T cells) with upregulated ILEI genes (interleukin-like epidermal-to-mesenchymal transition inducer) produce TGF-β [755]. Colon cancer cells (*vide supra*) undergo EMT and express the molecular signatures of up-regulated vimentin, E-cadherin, Slug and Claudin protein families, forkhead transcription factors, and WNT signaling with down-regulated dickkopf (*vide supra*) [757]. This author proposed that ras-mutated colon cancer cells were induced by T/T T-like lymphocytes, which promote their metastases in the regional lymph nodes [642]. Tumor cells (pancreatic carcinoma cells) expressing FoxP3, thus imitating T_{reg} cells, neutralize, and protect themselves from the attack of, immune T cells [758]. While tumor cells undergo EMT, the tumor stroma (the extracellular matrix) either plays the role of the initiator, or changes in response. Non-coding RNAs (miR-200; miR-205) interact with vimentin in the stroma and with E-cadherin within the tumor cells. TWIST protein induces miR-10b, which drives breast cancer cells' invasiveness, whereas miR-335 antagonizes the process [759]. The mammalian cell microRNA, miR-146a, emerges as an inhibitor of innate immune reactions and a multiple tumor growth promoter; it is activated by NFκB [760] The tumor-promoting events of EMT are open to study, if the cascade of gene activations occurs within the tumor cell, or if a fusion of the epithelial tumor cell with a mesenchymal cell (as well documented in the case of melanoma and macrophages) resulted in the transfer of activated mesenchymal phenotype-inducer genes into the tumor cell from a mesenchymal cell of the stroma.

When human epithelial cancer cells express immunoglobulins (RAG1, 2; VDJ; IgA) [761], is it in the cells' own genome, where the genes that are usually kept silenced were activated in a germline fashion, or is it the result of a "natural hybridoma" (NH) formation that has had occurred? In the process of the original natural hybridoma (NH) formation, antibody-producing plasma cells fused with lymphoma cells [213, 762–766], a mesenchymal-to-mesenchymal cell fusion. Epithelial cells may fuse with lymphocytes or monocytes to gain pre-activated genes for a mesenchymal transition.

The original NH formation occurred in a murine lymphoma. The NH phenomenon was discovered by this author in the mid-1960s, understood clearly and reported in explicit terms that a mouse leukemia virus-carrier lymphoma cell fused with a plasma cell secreting antibody specific to the lymphoma cell as the lymphoma cell expressed retroviral envelope antigens. This cell line (Fig. 2.4a,b)

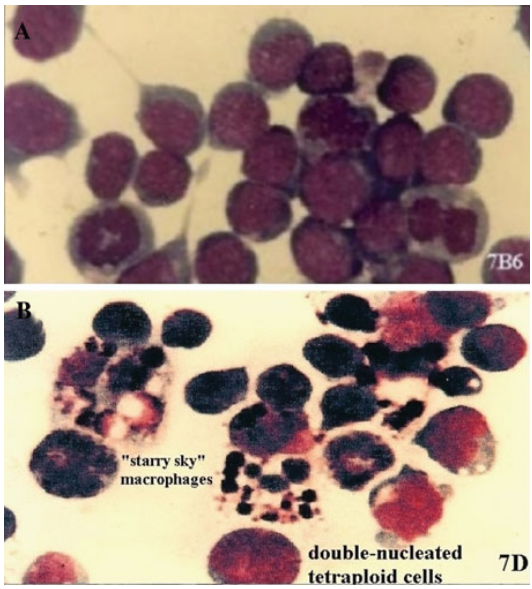

Fig. 2.4 (a,b) Spontaneous cell fusions in murine lymphoma observed in the mid-1960s at the Section of Clinical Tumor Virology & Immunology, M. D. Anderson Hospital, Houston, TX. The retrovirally transformed lymphoma cells showed budding retroviral particles (retroviral envelopes) in their cell membrane. The immune B (plasma) cells produced immunoglobulins specifically reacting with structural proteins of the virus particles budding from the lymphoma cells. The immunoglobulins neutralized infectious retrovirus in a spleen focus assay. The lymphoma cells and the immune plasma cells adhered to one another and fused. The fused products were tetra- or polyploid, grew in suspension cultures for over 10 years (**a**) and in the peritoneal cavity of mice, secreted the specific immunoglobulin, and were attacked by macrophages inducing the "starry sky" phenomenon (**b**). [767] The native spontaneous cell fusion event was duplicated in the peritoneal cavity of mice. Co-inoculated mixtures of lymphoma cells and immune plasma cells fused and produced bi-nucleated cells (**b**) [762–772]. In his first report on these fused cells, the author wrote in Lancet: "Tetraploid immunoresistant lymphoma cells in the mouse emerge by fusion of the diploid virus-producing lymphoma cell with a plasma cell producing virus-specific globulins. The resulting tetraploid cell will retain malignant growth potential and the genetically determined committedness of both parent cells – to produce leukemia virus, as coded for by the viral genome within the neoplastic cell, and to synthesize virus-specific globulins, as coded for by the genome of the plasma cell" [213, 765]. The USA National Cancer Institute replied in the mid-1970s to the author's grant applications: "approved without funding, due to low priority." The circumstances of this work were investigated, validated and credited for priority by Professor Milton Wainwright, University of Sheffield, Sheffield, England [819, 820]. Permission to re-publish is from Schenk Buchverlag, Passau and Budapest

was maintained in suspension cultures and as more ascites tumors for over 10 years, while it continued making the specific antibody [213, 762–772]. This author promptly proposed that the Reed-Sternberg cells of Hodgkin's disease may be B-cell natural hybridomas, and the Sézary cells of mycosis fungoides are T cell natural hybridomas [765, 769]. Molecular biology of RS cells so far provided no support for this theory, but there remains room for further reasoning in favor of natural hybridoma formation mediated by a fusogenic retrovirus (with or without EBV) in these pathological entities [213, 768–770].

When epithelial cells fuse with mesenchymal cells (EMT), the mechanism of fusion is not an explicit antigen-antibody reaction. The entire process of transdifferentiation of bone marrow stem cells may occur within the stem cell genome without fusion, by gene re-programming. In inflamed tissues, transdifferentiation of the stem cell may take the direction toward a malignant geno-phenotype [773]. In treated cancers, new stem cell type emerges; these are the "recurrent cancer stem cells"

(rCSCs). The rCSCs display strong resistance toward external chemo-radiotherapy by gaining strong anti-apoptotic faculties, and toward internal host immunity, thus practicing "oncogenic resistance." The phenotypic heterogeneity of these newly emerged rCSCs gives them the propensity to fuse with mesenchymal cells (monocytes) for their further advantage to invasiveness and metastasis formation [774].

2.8 Horizontal Transfer of Proto-Oncogenes

Peyton Rous at the Rockefeller Institute in New York City in 1908–1912 discovered tumor-induced neoangiogenesis, a monumental discovery, but not remembered, because of his another monumental discovery, that is, the Rous sarcoma virus. In 1908, the rapid reproducibility of lymphocytes upon stimulation caught Rous' attention [775]. In 1910 Rous wrote: "The fate of implanted tumor depends directly on whether it elicits from the host a vascularizing stroma. So, too, it is with implanted embryo" [776]. In 1910–1911, Rous transferred with cells and with filtrates a sarcomatous tumor in chickens (hens): the transmissible avian neoplasm, a sarcoma of the common fowl [777]. In 1912, Rous and Murphy observed lymphocytic activity in response to the transplanted sarcomatous tumors in the inoculated hens [778]. The Rous sarcoma virus and its sub-strains were the teachers of tumor retro-virologists world-wide for decades to come. It was the three editions of Ludwik Gross' textbook that provided the basic foundations of tumor virology for the early decades of that discipline [779]. Hidesaburo Hanafusa of the Rockefeller University, New York City, found the oncogenic genome of the Rous sarcoma virus (*v-src*) and the phosphorylated tyrosine kinases that it encodes in order to build the structural proteins of the virus particle; especially the *env*/Env gene and protein that was immunogenic. He realized that a cellular homologue of the viral oncogene existed (*c-src*) in the hosts of these tumors [780]. However, ahead of him at the University of California, San Francisco, the team of J. M. Bishop and H. E. Varmus, D. Stehelin and P. K. Vogt, established the DNA identity of the Rous sarcoma (and other avian sarcoma) proviruses in the cells of healthy hosts with that of the mature virions [781, 782]. The enzyme, the reverse transcriptase, which transcribes an RNA viral genome into a DNA provirus, was discovered by D. Baltimore and H. M. Temin and S. Mizutani [783]. Here is the most ostentatious (éclat!) example of gene switching: a host cell gene becomes incorporated into the DNA proviral genome of a retrovirus. It may remain independent within, or it may be fused with, a genomic sequence of the retrovirus, but when the retroviral genome is transcribed and translated, the usurped host gene and its gene product protein remain incorporated in the retroviral particle and expressed in the cell, respectively, that the virus transformed. If the usurped host gene encodes a growth factor, the retrovirus replicating in a malignantly transformed cell will overproduce that growth factor. Viral oncogenes and oncoproteins have been generated by gene transfer from a cellular genome into a viral genome.

While from fish to mammals (gibbon ape; woolly monkey) acute leukemias and sarcomas are caused by retroviruses, in the human host only one such retroviral pathogen could be isolated: the human T cell lymphotropic leukemia virus (HTLV) [784, 785].

In the 1970s, this author was engaged at the M. D. Anderson Hospital, Houston, TX. in efforts to isolate human sarcoma retroviruses. Cell free human sarcoma extracts and culture fluids could induce cell foci and antigenic conversions in human embryonic fibroblast cultures with occasional retroviral particles sighted, but up to this date no established human sarcoma virus could be isolated and identified. Human sarcoma cells certainly express "neoantigens" thus attracting cytotoxic lymphocytes (Fig. 2.2a,b). It is a matter of speculation how the hominoid genera and species (from *Australopithecus afarensis* to *Homo sapiens*) during their evolution succeeded in suppressing the leukemogenic and sarcomagenic retroviruses of their distant ancestors [213]. However, in Kaposi's sarcoma next to its causative herpesvirus (HHV-8) buds a reactivated endogenous retrovirus (Fig. 2.3a,b) [213].

Retrotransposons and the permanently inscribed genomic sequences (reversely transcribed DNA proviruses) of endogenous retroviral elements permeate the entire human genome, as relics of retroviral infections subdued in the distant and recent past. These are the viral genomes that may incorporate, propagate and horizontally spread genuine host cell genomic segments. The results of past and present endogenous retroviral activates extend from the evolution of the placenta and the telomeres, through the induction of autoimmunity and the reactivation and expression of certain endogenous retroviral elements in certain human cancers, the invocation of RNA interference and the tumor suppressor protein p53, to the ultimate primate evolution, including the development, present and future activities of the human brain [786–794].

2.9 Horizontal Gene Transfers for the Treatment and Cure of Cancer

The gene therapy of cancer was initiated by the replacement of the mutated or eliminated tumor suppressor genes (or whose gene product proteins were ubiquitinylated) by horizontal insertion of the wild-type genes. First, severe combined immunodeficiency (SCID) could be treated with bone marrow transplants. Haploidentical donor bone marrow transplants often induced graft-versus-host disease (GvHD) and failed to completely restore the function of B lineage lymphocytes. In clinical trials, children (infants) with adenosine deaminase deficiency (X-SCID) receive infused autologous bone marrow progenitor stem cells, which were transduced by a retroviral vector in vitro replacing the deficient gene. Restoration of the immune system occurs without GvHD [795]. In X-SCID the gamma c chain of the IL-2R is mutated (γ-c null) and is not signaling after stimulation with EBV. The cells with mutated IL-2R could be restored to function with a retroviral vector (G1γcSvNa) transducing the wild-type γ-c gene (c for common, because the IL-4R and IL-7R are also involved). In the transduced cells, the IL-2Rs signaled normally and phosphorylated the Jak1/Jak3 (janus kinase) tyrosine kinases [796]. However gene re-insertions by a murine leukemia retroviral vector carried a not foreseen major risk. Five of 20 patients developed acute T cell leukemia (first reported as "clonal T cell proliferation") after retrovirally vectored gene insertion. The vector retrovirus inserted its genome carrying the correct gene next to the T cell proto-oncogene LMO2, and thus activated it by the mechanisms of "insertional oncogenesis" [797–799]. The proto-oncogene LMO2 stands for "LIM only protein 2" and LIMs are mesenchymal (muscle, etc) proteins encoded by a family of genes (*lin/isl/mec*). The LMO2 gene is involved in T cell generation and in its activated state transforms from a proto-oncogene into an oncogene [800]. A single chain Fv antibody fragment was developed to specifically inhibit LMO2 [801], but its clinical usefulness is not yet known.

The Max Delbrück Institute of Molecular Medicine, Berlin, Germany, has taken the directives toward nonviral delivery approaches in human gene therapy. A plasmid expressing a transposase in *trans* position and a donor plasmid containing the DNA (gene) to be integrated are to be used in combination. The DNA to be integrated is flanked in cis position by the terminal repeat sequences of the transposase. The candidate transposons are the Tc1/mariner-type *Sleeping Beauty* (SB), the *Tol2* (both originally fish transposons) and the *piggybac* (PB). In order to increase the activity of SB, the transposase had to be reconstructed by exchanging its aa composition. Thus arriving at the hyperactive SB100X transposon/transposase, its increased activity in its integration process is over one hundred-fold elevated. The nonmariner-type Tol2 transposon can carry genes as large as 11 kb. While excess transposase could inhibit SB by "overproduction inhibition," Tol2 works better with more transposase at its disposal. The PB transposon is of insect origin (deriving from the lepidopteran noctuid, the cabbage moth, *Trichoplusia ni*). It was discovered because it inserted itself into the genome of the nuclear polyhedrosis virus, the baculovirus *Autographa californica* [802–804]. PB can transpose inserts of 14 kb (too large for any retroviral vector). While SB has no human relatives, PB has its related sequences dispersed in human chromosomes. It is a concern how these endogenous human PB

elements would react to the insertion of an exogenous PB transposons. The LTR sequences of SB100X are inert when it comes to activating host cell genes at the transposons integration site. This inactivity has been reinforced by adding an insulator sequence to the expression cassette of the transposons. In contrast, N-terminal DNA-binding domain fused with the transposon PB rendered the transposase more permissive as to its acceptance of DNA (gene sequences) for integration. Silent pluripotent stem cells also accept genes integrated by transposons for the re-programming of these cells. In induced pluripotent stem (iPS) cells, the c-*myc*× c-Myc activation carries the potential danger of oncogenesis. Some called c-*myc* "the oncogene from hell." Therefore activation of c-*myc* should be avoided (especially in the case of retroviral vectoring). The PB transposon could already safely deliver genes into mouse fibroblasts and thus reprogram them into iPS cells of endodermal, mesodermal and ectodermal lineages. The natural transposons/transposases become "designer transposases" opening up avenues toward regenerative medicine and cancer therapy by horizontally inserted genes [805].

After proving in mice that SB100X-inserted reporter gene-carrier hematopoietic stem cells function impeccably, human clinical trials have been initiated. In the first human clinical trial, SB100X will encode a chimeric T cell receptor for adoptive immune T cell therapy in patients with $CD19^+$ B lymphocyte lineage malignancies (malignant lymphomas). The new T cell receptor will consist of a specific CD19-reactive single chain v fragment linked to the CD28 endodomain which is fused with the cytoplasmic CD3-zeta (ζ) domain. It was already proven that T cells so reconstructed are cytotoxic to $CD19^+$ B lymphoma cells. The cytotoxic T cell clone will be expanded in vitro on irradiated $CD19^+$ lymphoma cells constantly stimulating it. Genetically engineered T cells will be infused during the period of recovery from myeloablative chemotherapy and autologous stem cell rescue [805–816].

2.10 Consequences

The once believed accidental, and exceptional horizontal (lateral) gene transfers and cell fusions have become accepted as one of the absolute rules of Nature. So much so, that when some vertically inherited genes have been mistakenly claimed to be horizontally acquired; the matter was quickly corrected [817]. Excessive exchange of genes outside of the vertical route by whatever other means (protocell fusions; protocell fusions fusogenic bacteriophage-mediated, naked DNA transfers; plasmid-, phage- and virus-mediated transfer) were the fundamental attributes and inherent characteristics of the living matter. The voracious acquisitions of alien genes in protocells, and in their immediate descendants prohibit a firm rooting of the Tree of Life below the Darwinian threshold.

To this author, the genome of the neoplastic cells resembles most the ancient wild-type premordial DNA: aggressive, an eager host (predator) of engulfed genes, and a most willing partner in gene fusions. It is immortal: it divides before its senescence (telomeric loss) and death could set in. Whereas the DNAs serving in multicellular hosts must have undergone through several "taming mutations" resulting in the acceptance of differentiation, service to the cell community, then senescence and death. However, within the stem cell compartments, silent remnants of the ancient wild-type DNAs prevail. In a blind rebellion, the stem cell DNA may divest itself from its superimposed role to differentiate and serve. Instead, it initiates a rebellious attempt at regaining its ancient immortality. The clinicians at the bed side diagnose the formidable disease: "cancer." However, the clinicians are not observing a "disease" as such. The inherent archaic DNA is rising to re-occupy its native territory, expand in the oceans and land as long as nutrients are provided and wastes are eliminated. If the environment freezes in the absolute temperature of the outer space, the DNA waits a milliard years until after it reaches another environment appropriate to its divisions, mutations, fusions and expansions. In another Cambrian sea, the tamed DNA-derivatives begin their service within multicellular organisms, while harboring in their sequences some silent remnants of their wild-type ancestry. "Cancer" is not a

disease as such; it is the ancient wild-type DNA's inscribed and inherent faculty for its immortality in any shape or form under any circumstances.

The explosive evolution culminating in the armada of creatures in the Cambrian sea, slowed down horizontal gene transfers under the newly installed rules and regulations of vertical inheritance, in which the retention of point-mutated, amplified, recombined and duplicated genes represent the driving force. From this point on, there is a clearly recognizable Tree of Life. Even those highly disciplined cells that form the Tree of Life would not refrain from accepting a useful alien gene whenever a rare opportunity still arises. In other instances, an uninvited retrotransposon or a virus will intrude and implant a new gene into a rigidly organized genome. The host, its defenses overcome, tries to reduce these sequences to pseudogenes, or actually takes use of them as new useful genes; other inserted sequences remain inert for millenia. F. Bushman provided a tabulated list of human genes potentially derived from transposable elements (including telomerase, human endogenous retroviruses, placental syncytins and *rag*s 1 and 2) [435].

Provoke the genomes of plants, domesticated animals and human beings living on Mars: the discipline of the rigidly regulated genomes will melt. In response to the challenge, the genomes will re-open the gates for the acceptance of mutations, gene duplications and new genes. That would be the course of natural evolution. However, gene therapists will intervene to produce a heavy set human race on a planet with low gravitational force, with allowances to anaerobic (Warburg) glycolysis, and with the lipid metabolism of the arctic (polar) bear, the seal (or the penguin). Processes of the lateral (horizontal) gene transfers from archaea and prokaryota to eukaryota and from eukaryota to multicellular eukaryota, substantially restricted, but remaining active, will continue as long as cells evolve on Earth (or on Mars).

2.11 Summary

Voracious lateral (horizontal) exchanges of alien genes between the ancestor proto-spheroplasts of archaea, prokaryota and the first unicellular eukaryota, occurring below the Darwinian threshold, render the rooting of the Tree of Life close to be impossible. A fusogenic phage might have mediated the primordial fusion between crenarchaeal and prokaryotic protoplastic cells to form the first ancestral eukaryotic cell(s). Descendants of fusogenic mycoplasma phages and extant proto-spheroplasts of crenarchaeota and prokaryota may be able to repeat this seminal experiment of Nature in the laboratory. If not by free-swimming DNA retrotransposons encoding the genes for the enzymes that carried out insertions and excisions, horizontal gene transfers were mediated by viruses. Plasmids and bacteriophages served as ancient vehicles of laterally transferred genes. The ancestors of the large dsDNA cytoplasmic viruses (the mimivirus and its relatives) contributed to eukaryogenesis in the "Virus World." These very large viruses remain in existence through some 3 billion years as parasites and/or symbionts to the descendants of the first unicellular eukaryotes. Above the Darwinian threshold, as speciation and the rules of vertical inheritance established themselves, horizontal gene transfers gained another route: the cell fusions, whether hostile or symbiotic, promoting the exchange of alien genes. Some of the most important genes encoding the adaptive immune system in the first chondrichthyes sharks in the Cambrian sea were acquired through horizontal insertions The reticuloendothelial retrovirus inserted its genome into Marek's herpesvirus, or into the fowl pox virus. There is a "criminal collusion" between herpes- and retroviruses dating back to tens of millions of years of co-evolution in the same hosts (simians, primates, *Australopithecines,* hominoids and *Homo*) in Africa.

How did the original cholera vibrio acquire its cholera toxin genes? What is the oceanic or estuarial environment from which the cholera vibrio and its phages emerged? The donor of the cholera toxin genes (blooming zooplanktons; a dinoflagellate; a crustacean) swims free and unrecognized, while the cholera vibrio spreads around the globe. Of ancient marine mycobacteria, the genes that

encode the mycolactone toxin that causes the Buruli ulcer might have been acquired from an ancient amphibian (a frog) by the ancestor of *M. marinum* and passed by plasmids from it to the later becoming human-pathogen *M. ulcerans*. The genes encoding antibiotics and antibiotic-resistance, and those of "pathogenicity islands" appear to have emerged after speciation evolved, above the Darwinian threshold. Nevertheless, these gene clusters remain the subjects of horizontal transfers between prokaryota.

Eukaryotic cells, including those of the human brain, accept horizontally inserted genes (viral genes, such as those of the Bornavirus). A review with selected references is provided on horizontal gene transfers from archaea and prokaryota to eukaryota and from eukaryota to eukaryota. The genes (operons) of photosynthesis were transferred from cyanobacteria to algae through lateral routes (from engulfed cytoplasmic chloroplasts to the host cell nucleus) and from there to terrestrial plants by vertical inheritance, or into the apicoplasts of plasmodia and other apicoplexan unicellular eukaryota from red algae by horizontal transfers. The female wasps demonstrate superb ingenuity in converting pathogenic ascoviruses into, non-pathogenic in-the-wasps, ichnoviruses, which are immunosuppressive in the caterpillars. There, the hatching of the wasps' fertilized eggs after their insertion culminates without immune rejection. Some insect cell nuclei carry from generation to generation the entire genomes of rickettsia-like bacteria (Wollbachia) and exchange genes with them. The insertion of cellular proto-oncogenes into retroviral genomes (proviral DNAs) is the most ancient form of natural oncogenesis. Inflammation-induced carcinogenesis is analyzed against the backgrounds of chronic bacterial infections with *Helicobacter pylori* and *Bacteroides fragilis*, and that of epithelial-to-mesenchymal transformation of pre-cancerous stem cells and of cancer cells. In cancerous tissues "natural hybridoma" formations may occur by fusion between lymphoma cells and antibody-secreting plasma cells (first observed, understood and reported in 1970 by this author), or between epithelial cancer cells and mesenchymal (lymphocytes, monocytes) cells. Of these, fusion of human melanoma cells with host monocytes/macrophages the most ostentatious example; here again, a reactivated endogenous retrovirus in the melanoma cell may be the fusogen. Gene therapy with horizontally transferred tumor suppressor genes utilizing transposons as vehicles may induce remissions in, or even cure of, human cancers.

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