Cell Signaling During *Trypanosoma cruzi* Development in Triatominae

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Abstract: The year 2009 is the centennial anniversary of the original description of Chagas disease by Carlos Chagas. During the last 100 years, several advances have occurred regarding our knowledge of the development of *Trypanosoma cruzi* and its travel along the gut of Triatominae vectors. We have also witnessed the completion of both the human and parasite genome projects; the genome of one of Chagas disease vectors, *Rhodnius prolixus*, is currently being sequenced. The development of *T. cruzi* in triatomine gut relies on several biochemical and molecular processes. The biochemistry of blood digestion and the molecular and biological aspects of parasite development are well known. However, several signaling molecules are generated during blood digestion, and their effects on parasite biology are only beginning to be understood. Here, we will summarize our current knowledge in this area with an emphasis on heme and bioactive lipids. In addition, we will highlight some recently described members of the parasite signaling machinery, which were identified through high-throughput studies, but whose ligands are unknown thus far. Finally, we will discuss some potential aspects for future investigation in this area that may strengthen our view of such a concomitant biological process in the next years.

Keywords: *Trypanosoma*, heme, lysophosphatidylcholine, Chagas disease, *Rhodnius*.

INTRODUCTION

Chagas disease is caused by the trypanosomatid protozoan *Trypanosoma cruzi*. This disease was first described in the early twentieth century by Carlos Chagas, who reported its pathophysiological aspects and mechanism of transmission in 1909 [1-3]. Unfortunately, 100 years later, specific and efficient methods to block the transmission of this parasite and treat this condition still remain controversial [4]. The current number of patients infected with this disease is 11 million, with 200,000 new cases and at least 21,000 associated deaths each year [5].

The digestive system of Triatominae is generally composed of a pair of salivary glands, the anterior midgut (crop), the posterior midgut (small intestine) and the hindgut (rectum) [6]. After a meal, the ingested blood is stored in the crop and water is rapidly excreted by the Malpighian tubules [7]. Diuresis occurs early in the digestive process and concentrates the meal, which is slowly released from the anterior into the posterior midgut where enzymatic digestion occurs. The passage of the meal to the lumen of the posterior midgut is accompanied by the secretion of proteases, which are well characterized as amino peptidases, carboxypeptidases, cysteine proteases and aspartic proteases [8]. After a triatomine bug feeds on an infected mammalian host, the ingested trypomastigotes transform mostly into epimastigotes and some into spheromastigotes in the (triatomine) anterior midgut. In the posterior midgut, epimastigotes attach to perimicrovillar membranes (PMM), which are secreted by midgut epithelial cells, and divide by binary division. This membrane system covers the midgut microvilli and extends towards its lumen [8].

Finally, once at the rectum, epimastigotes weakly attach to the hindgut cuticle and transform into metacyclic trypomastigotes, which are expelled with triatomine urine and feces and are then able to infect mammalian hosts [9-12].

The process of parasite differentiation relies on the attachment of parasites at different sites along the gut tissues of the vector as well as on the influence of soluble gut contents, whose constitution varies immensely along the course of the blood digestion process [12-13]. The attachment of *T. cruzi* epimastigotes to the PMM has been suggested to be important for parasite division [9]. Recently, the involvement of glycoinositolphospholipids (GIPLs), the major surface glycoconjugates of *T. cruzi* epimastigotes, in the adhesion of the parasite to the posterior midgut was demonstrated [14]. Despite the largely studied effects of GIPLs on vertebrate immune cells, the signaling pathways affected in the insect have not yet been studied. Adhesion of epimastigotes to the rectum surface is thought to be a prerequisite for differentiation into its infectious stages and involves hydrophobic interactions with the superficial cuticle layer of the hindgut [15-16]. It is not clear how adhesion triggers metacyclogenesis, but it must involve signaling pathways mediated by parasite surface receptors, which are activated by ligands present in vector tissues. In this respect, some glycoproteins present in the PMM seem to be parasite ligands, but their identities remain to be determined [17]. As blood digestion proceeds, both adhesion and metacyclogenesis *per se* are elicited by nutritional stress, which induces the expression of several stage-specific genes associated with the differentia-

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tion mechanism through a still unknown signaling pathway [18].

Establishment of a *T. cruzi* infection in an insect vector is dependent on and regulated by several physiological and biochemical factors (Fig. **1**). Parasites enter the insect gut with the blood meal. In this context, they are submitted to drastic changes in temperature, pH and osmolarity and are exposed to saliva, gut contents and blood digestion products [12]. Some factors involved in the development of *T. cruzi* in the gut of the vector have been identified, including a lytic factor [19, 20], lectins [21, 22] and hemoglobin constituents [23]. Other factors are suggested to trigger *T. cruzi* metacyclogenesis *in vitro*, including cAMP, cAMP analogues and adenylyl cyclase activators. Furthermore, intracellular levels of cAMP increase three- to four-fold before differentiation from epimastigotes to metacyclic trypomastigotes occurs [24]. In *Rhodnius prolixus*, cAMP excreted by Malpighian tubules and present in the urine might play a direct role in metacyclogenesis *in vivo* [25]. Vector hemolymph and intestine-derived components are able to promote metacyclogenesis as well. For example, an α-D-globin-derived peptide isolated from *Triatoma infestans* is recognized by epimastigote surface receptors and stimulates parasite adenylyl cyclase, which leads to metacyclogenesis *in vitro* [26]. Subsequently, the effects of hemoglobin and synthetic peptides carrying α-D-globin fragments on parasite growth and metacyclogenesis have also been demonstrated *in vivo* [27]. Therefore, the generation of biochemical mediators during blood digestion is clearly a central aspect of parasite development, as well as its growth and infection. In this review, we will emphasize signaling mediators derived from blood digestion that are involved in the regulation of the parasite life cycle in the vector.

Triatominae ingest five to ten times of their own weight in blood in a single meal [27, 28]. Hemoglobin digestion in the posterior midgut may induce an increase in heme concentration of up to 10 mM (Fig. **1**). In addition to forming reactive oxygen species, free heme can associate with phospholipid membranes to alter the structure of the lipid bilayer, leading to cell disruption [29, 30]. Fenton's chemical reaction is a prime example of a harmful free radical reaction that is catalyzed by transition metals [31]. During Fenton's reaction, hydrogen peroxide, in the presence of iron (II), generates a highly reactive free radical species, the hydroxyl radical. Free heme creates a particular source of oxidative stress that may influence signaling pathways in surrounding cells. A close look at the antioxidant defense system in *R. prolixus* exposed the presence of superoxide dismutase (SOD), which catalyzes the dismutation of the superoxide radical (O_2^-) [32], shows that this reaction produces hydrogen peroxide, which is, in turn, degraded by catalase (CAT). The highest SOD and CAT activities among all tested tissues were found in the posterior midgut. Injection of the CAT inhibitor 3-amino-1,2,3-triazole (AT) promoted a dose-dependent inhibition of enzyme activity, with the concomitant production of high levels of hydrogen peroxide. Direct measurement of the hydrogen peroxide in this system was achieved in AT-treated insects. These results suggest that scavenging systems are important for antioxidant defense in the intestine. In this specific compartment of the vector digestive system, the processes of blood digestion and parasite differentiation are very advanced. It is tempting to speculate that the parasite must also display similar antioxidant defenses in order to face the redox environment of the intestine. However, such defenses have not yet been demonstrated.

Epimastigotes of *T. cruzi* actively multiply in the anterior midgut of the vector. Sequencing of the genome of this parasite revealed the absence of eight enzymes of the classical glycine-based heme synthesis pathway [33]. Heme-, hemoglobin- and globin-derived peptides were shown to induce parasite proliferation [34]. In the parasite, heme internalization probably occurs with an ABC transporter and in a region concentrated to the posterior end of the parasite that corresponds to reservosomes [34]. Reservosomes are phospholipid, ergosterol and cholesterol storage organelles and are localized to the posterior region of the epimastigote form of *T. cruzi* [35]. The content of these organelles is consumed during metacyclogenesis. It is likely that heme, as a lipophilic molecule, may induce some degree of lipid peroxidation as it is transported to the parasite and, once lipid mediators are generated, may influence parasite proliferation. While studies have shown that heme induced-signaling on parasite proliferation is solely mediated by calcium-calmodulin kinases (CaMKs), the role of a redox-mediated mechanism in this process has still not been assessed [36].

Studies in eukaryotes have determined two main families of proteins directly involved in signaling responses under redox stress: protein kinase C (PKC) and protein tyrosine phosphatase (PTP). PKC is a family of Ser/Thr protein kinases and is composed of at least 10 different isoforms. Its involvement with heme-induced oxidative stress was originally demonstrated in the regulation of the production of the anti-oxidant molecule urate by the fat body in *R. prolixus* [37]. Urate production is induced by cAMP and blocked by protein kinase A (PKA) inhibitors. Curiously, in the presence of heme, agonists of the cAMP pathway do not exert the same effects. In this situation, PKC is the enzyme responsible for the regulation of antioxidant production. However, the role of these pathways has not yet been tested in midgut epithelial cells, and the mechanisms, by which they regulate PKA- and PKC-mediated phosphorylation during blood digestion, if any, are not currently known.

The second family of signaling enzymes that may be involved in the regulation of cell signaling under redox conditions is the PTP family. These enzymes are involved in several intracellular signaling events, such as cell adhesion to substrate, cell metabolism and gene expression [38]. These enzymes are grouped into three different classes: protein tyrosine phosphatases (PTPs), Cdc25 and low molecular weight phosphatases (LMW-PTPs). They share a common motif (CX5R) in their catalytic site that allows for a cysteine-based catalytic cycle. In humans, the PTP group is subdivided into classical PTPs and dual specificity phosphatases (DSPs). Classical PTPs may be either receptor-type or non-receptor-type. The unique catalytic reaction involving the SH group in the catalytic site implies possible modulation of PTPs by pro-oxidant molecules. Several redox states have been determined for these enzymes, which are strongly dependent on cell environment. Our group demonstrated that, with respect to blood-sucking arthropods,

PTP regulates protein degradation during tick egg development [39]. Modulation of PTP gene expression occurs in some systems. Parasites traveling through the vector digestive system may be affected by redox-mediated signaling through both PKC and PTPs. A developmental control of surface ecto-phosphatase activity seems to occur in the epimastigote stage of *T. cruzi*, which displays the lowest enzyme activity in all tested stages [40]. Modulation of ecto-phosphatase activity at the *T. rangeli* surface by hydrogen peroxide has already been demonstrated [41]. Furthermore, it was demonstrated that Fenton's reaction affects the activity of another signaling molecule from the parasite's surface, an ecto-nucleoside triphosphate diphosphohydrolase in *T. brucei brucei*, the causative agent of African trypanosomiasis [42]. These studies revealed that the redox environment may finely tune the intracellular environment through the modification of signaling enzymes both in the vector and at the parasite surface. However, similar studies regarding vector and parasite PTPs, and surface enzymes of *T. cruzi* have not yet been conducted.

Typically, the average lipid concentration in vertebrate blood serum is 800 mg/dL [43]. Therefore, in a single blood meal a fifth instar bug may ingest close to 1 mg of lipids which is equivalent of a 70 Kg human to ingest close to 1 Kg of lipid daily! Thus, in a typical blood meal, an adult bug or a fifth instar nymph may ingest approximately 2 mg of lipids. The posterior midgut of the blood sucking triatomines is a major site of dietary lipid absorption. The digestion of neutral lipids, phospholipids and glycolipids generates a proportionally larger amount of free fatty acids that, upon absortion by midgut epithelial cells, will be used for *de novo* synthesis of diacylglycerols, triacylglycerols and phospholipids. The midgut provides lipids to be used in insect tissue development. The function of a hemolymphatic lipoprotein, lipophorin, distributes lipids to different organs, including the ovaries in order to attain their lipid demand during egg formation [44-46]. Usually, the complete digestion of a blood meal occurs over 12 days in Triatominae. Ten days after a blood meal, the digestion is almost complete, and there is a shift in the lipid metabolism in the posterior midgut. Injections of radiolabelled oleic acid (OA) have shown that the posterior midgut is the main organ that incorporates this lipid [46]. Furthermore, OA is incorporated into phospholipids and neutral lipids. These data indicate that, in the earlier phase of blood digestion and absorption, the insect posterior midgut provides lipids to the surrounding organs, but as digestion slows, the lipid flux is reversed and the posterior midgut receives lipids from the hemolymph [46]. It is possible that some of these lipids are incorporated into the PMM during their synthesis, which occurs at the initial phase of digestion (Fig. **1**).

Trypanosomatids have incomplete *de novo* lipid synthesis and usually absorb lipids from the vertebrate bloodstream in order to meet their growth and differentiation requirements. Therefore, these pathogens critically rely on the presence of vertebrate lipoproteins, especially vertebrate LDL, to achieve infection. Despite this general and classical view of lipid metabolism in protozoan parasites, it is important to note that some differences may occur in the pathway with regard to cholesterol. Promastigotes of *Leishmania* synthesize a large amount of their sterols from leucine. *T. brucei* acquires phospholipids and sterols through endocytosis and intracellular processing of host LDL [47]. We have previously shown that *T. rangeli*, a trypanosomatid that reaches the vector hemolymph, uptakes the major hemolymphatic lipoprotein, lipophorin (Lp), from *R. prolixus* [48]. Also, we have shown lipid uptake by the malaria parasite during its life cycle in the invertebrate host [49]. Molecular evidence for the role of Lp in malaria parasite development has been provided [50]. However, the direct uptake of Lp and lipids by malaria parasite stages while inside its vector are not available. The published data provides evidence for the interaction of protozoan parasites with the lipid sources available in different insect environments. In this respect, the digestive system of triatomine bugs is a rich source of these molecules. Considering that these insects usually feed on huge amounts of blood, a massive amount of lipids is simultaneously ingested with trypanosomatids. A direct demonstration of such a pathway is still lacking for *T. cruzi*-infected vector.

A PKC from *T. cruzi* epimastigotes was characterized, and its biochemical and immunological characteristics have been analyzed [51]. The involvement of this enzyme in sensing the lipid environment and its role in the parasite was revealed upon the demonstration that treatment of epimastigotes with intestinal homogenate from *Triatoma infestans* led to an increase in intracellular calcium followed by parasite differentiation to the trypomastigote stage [52]. Protease treatment of the intestinal fraction did not abolish the induction of differentiation. This effect is restricted to the lipid fraction of intestine, especially to OA. This fatty acid stimulates the *de novo* synthesis of diacylglycerol and the activation of PKC, which leads to parasite differentiation [53]. OA is able to trigger intracellular calcium in epimastigotes, inducing the translocation of several PKC isoforms, such as α, β, γ and δ. PKC inhibitors abrogated both parasite differentiation and the membrane translocation of various PKC isoforms [54]. It will be interesting, in the future, to test the effects of heme-mediated lipid peroxidation in this OA fraction, specifically for their ability to promote PKC regulation in parasite differentiation (Fig. **1**).

Some studies have concluded that the production of hydroxyl radicals can occur from free heme either in solution or bound to hemoglobin [55]. However, there is no evidence indicating that lipid peroxidation induced by heme occurs during blood digestion. Furthermore, lipid hydroperoxides may be formed during lipid peroxidation, which is initiated by another source of reactive species, such as O_2^- formed either by NADPH oxidase or during mitochondrial respiration. This fact suggests that the pro-oxidant action of heme can be best described as an amplification of previously formed reactive species and depends on the occurrence and magnitude of other sources of free radicals [56]. In conclusion, despite the presence of the antioxidant enzyme system described above, lipid peroxidation may occur as a result of high levels of free heme and production of radical species from other sources. It is strikingly possible that lipid peroxidation products could be observed, for example, upon the inhibition of heme detoxification systems, such as heme-oxigenase. This interesting point should be addressed in the future.

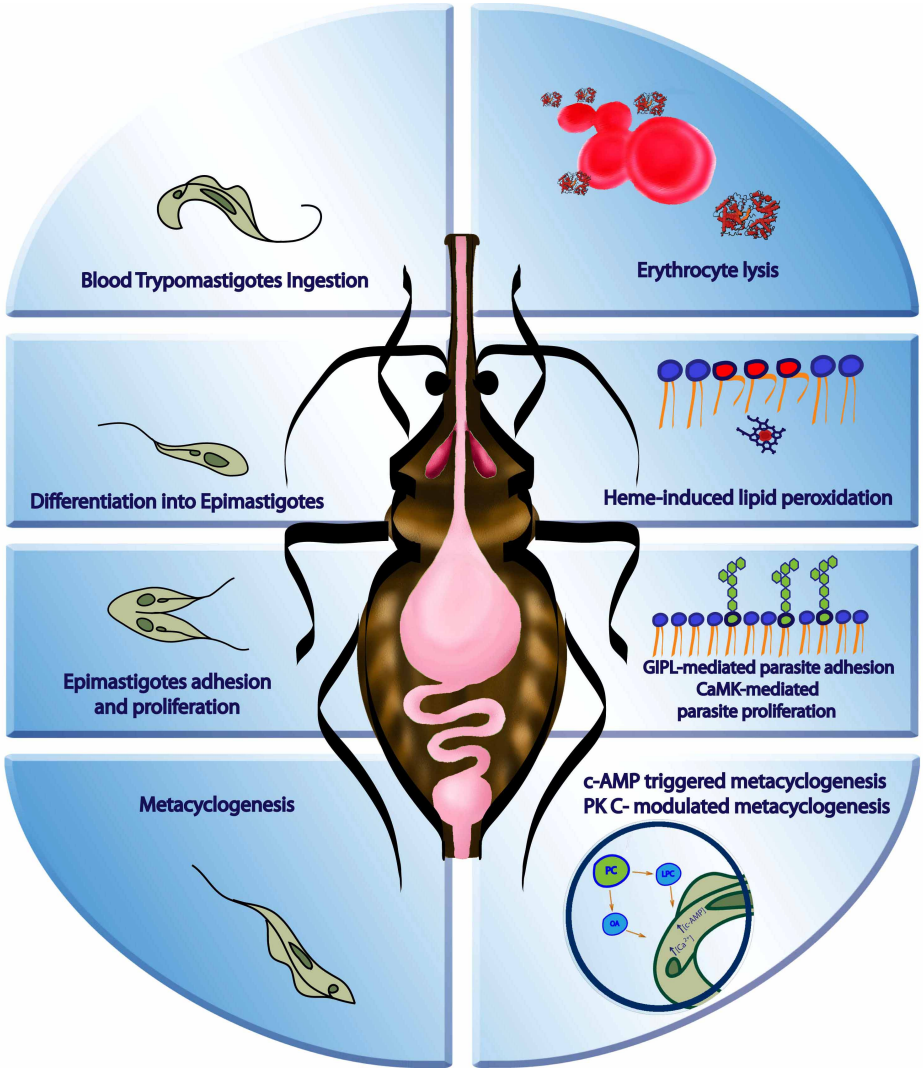

Fig. (1). Overview of major signaling events during *T. cruzi* development in Triatominae digestory system. *Left panels*, parasite development: *Right panels*, biochemical and signaling mechanisms. After a blood meal on an infected host, trypomastigotes transform mostly into epimastigotes and some into spheromastigotes in the anterior midgut, where following erythrocyte lysis, hemoglobin is gradually relased. Catalytic amounts of heme may attack unsaturated phospholipids from plasma lipoproteins producing several unknown lipid peroxidation products. In the posterior midgut, epimastigotes attach to perimicrovillar membranes through surface glycophosphatidylinositols and divide by binary division. Free heme modulates parasite proliferation through the activation of CaMK kinase. Finally, once at the hindgut, epimastigotes weakly attach to the rectal cuticle and transform into metacyclic trypomastigotes. Phospholipid-derived bioactive lipids such as oleic acid (OA) and eventually lysophosphophatidylcholine (LPC) regulate metacyclogenesis through generation of intracellular signaling molecules such as calcium and cyclic-AMP. Finally, parasites are expelled with triatomine urine and feces and are then able to infect mammalian hosts. The events in this cartoon have been already described in the literature and some of them are still under investigation. Please see the text for further details.

Usually phospholipids, mainly phosphatidylcholine, compose 40% of the ingested lipids in a blood meal. Blood feeding followed by the initiation of digestion may lead to the destruction of the blood cells and a release of large amounts of phospholipases, such as phospholipase A_2 (PLA_2). PLA_2 catalyzes the hydrolysis of the *sn*-2 fatty acyl bond of phospholipids, with the release of a free fatty acid and a lysophospholipid. Both of these products are important signaling molecules. We have shown the presence of lysophospholipids, mainly lysophosphatidylcholine (LPC), in the saliva and feces of *R. prolixus* [57,58]. LPC acts as a chemotactic molecule for monocytes and macrophages, but also plays a role in the production of NO by these cells, therefore enhancing *T. cruzi* infection through immunosuppression. Curiously, the phospholipase A1 was identified in the infective stages of *T. cruzi*, but not in epimastigotes [59]. This finding suggests that the parasite may rely on LPC that is derived from the insect, from the host and from its own enzymatic system. However, future tests on the ability of lysophospholipids derived from the parasite itself to immunosupress host cells as compared to that generated by PLA_2 are required. Fatty acids released upon PLA_2 hydrolysis, or present in the host blood and associated to albumin, may be processed by the eicosanoid pathway. Furthermore, proteomic analysis of epimastigote parasite forms identified the presence of prostaglandin F2α

synthase [60]. Prostaglandin PGH_2 can be converted to the eicosanoid thromboxane A_2 (TXA_2) by *T. cruzi*. TXA_2 functions as a key regulator of pathogenesis in parasite infection in mice [61]. However, modification of fatty acids by the eicosanoid pathway has not yet been evaluated in the vector stages of *T. cruzi* development.

Usually 10% of concluded kinomes code for lipid-modulated kinases. These enzymes are continuously regulated by diacylglycerol (DG) and phosphoinositides due to the presence of either a C1 domain that binds DG, or a pleckstrin homology domain (PH) or PH-like domain that binds 3'-phosphoinositides. Also, in PKC, some C2 domains may bind phospholipids in the presence of calcium [62]. Analysis of the *T. cruzi* kinome revealed the presence of 190 genes coding for eukaryotic PKs (ePKs). Trypanosomatids have about half of human kinases assigned to the AGC group. This group includes the ePKs that respond to second messengers, including phospholipids and diacylglycerol [63]. However, out of 12 genes only five genes could be assigned to a specific kinase family by sequence, and the remaining seven were not assigned to the PKC family. Due to the ability of the lipid environment to influence *T. cruzi* development, one should look at the structure of lipid kinases in detail. It is likely that some structural differences may be present in the lipid-binding motifs of trypanosomatid lipid kinases, which would provide a promising possibility for the future development of novel chemotherapeutics. Also, different groups have analyzed genes coding for PTPs, which has provided interesting data. The *T. cruzi* genome codes for only two intracellular, classical PTPs, fully dedicated enzymes for tyrosine dephosphorylation [64-66]. Therefore, if PTPs evolved independently several times, it is likely that protozoan enzymes display unique structural motifs or mechanism of regulation, which could allow for the development of specific chemotherapies [67].

We are living in the *T. cruzi* post-genomics era where a large amount of genomic information is available, facilitating *in silico*, proteomics and functional analyses of potential parasite targets for chemotherapy research against Chagas disease. In this context, drugs that act by modulating signal transduction pathways are particularly interesting. Although this matter remains largely unexplored in trypanosomatids, recent work has generated promising data [60, 63, 64, 68-70]. A comparative study of the kinomes of trypanosomatids *T. brucei*, *T. cruzi* and *Leishmania major* showed that approximately 12% of their kinases are unique to trypanosomatids [63, 69]. Among the protein phosphatases genes, about 40% are atypical, with no clear orthologs in other eukaryote genomes [64]. The significant differences between *T. cruzi* and host-cell protein kinases suggest that their specific inhibition may represent a viable therapeutic approach to control Chagas disease. Finally, phosphoproteomic analysis of the epimastigote form of the parasite led to the identification and mapping of 237 phosphopeptides from 119 distinct proteins [70]. The identified phosphoproteins are involved in cell structure, motility, transportation, metabolism, pathogenesis, DNA/RNA/protein turnover and signaling. These phosphoproteomic data provide new insights into the molecular mechanisms governed by protein kinases and phosphatases in *T. cruzi* [70]. The accessible data may now be assayed in terms of parasite biology.

Nevertheless, our understanding of the mechanism of *T. cruzi* growth and differentiation in kissing bugs, with the identification of all of the involved components, their mode of interaction and activated signaling pathways, is far from being complete. In this scenario, multidirectional cellular signaling modulators, either generated from blood digestion or produced by the interaction between the parasite and the vector itself, are strong candidates for the modulation of *T. cruzi* development. This model will provide a huge source of information that certainly will keep us busy for the next 100 years.

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