

Blood transfusion and the presence of biological structures in the circulation

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ABSTRACT

Advances in knowledge continue to be made regarding biological structures which may be present in blood circulation, such as circulating cell-free DNA, extracellular vesicles, neutrophil extracellular traps (NETs), and activated platelet-derived or circulating cell-free mitochondria. These circulating elements may be of systemic significance, in particular with respect to immunomodulation and cell-to-cell communication. This fact highlights the need to take into consideration the delivery to the host of various biological structures and by-products by means of blood- or blood products transfusion; and to investigate their potential side effects. The significance of these structures and their reported potential effects are discussed in this review. However, to date, no evidence of deleterious effect following blood or blood products transfusion was reported.

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Introduction

Advances in knowledge of blood composition continue to be made. The recent discovery of the presence of circulating cell-free respiratory competent mitochondria (1) highlights the need to take into consideration the delivery to the host of various biological structures and by-products by means of blood- or blood products transfusion. Over the past decade, the presence and physiological role of various biological structures (microvesicles, exosomes, circulating DNA, neutrophil extracellular traps (NETs) or mitochondria) in the circulation have been demonstrated (1,2). These facts provoke certain questions. For instance, do those structures have a biological effect after transfusion? If so, is that effect deleterious? To counter that possibility, should the already stringent blood and blood product quality controls be further enhanced?

Blood transfusion is one of the most widely used medical treatments (3). It may be needed for conditions that affect the red blood cells, such as Sickle cell disease or thalassemia; it may also be required during cancer or in instances involving severe bleeding, such as surgery, childbirth or traumatic accident. The most commonly transfused components are red blood cell units, transfusible plasma, and platelet concentrates (4). The transfusion of whole blood or granulocytes is rarely performed.

Blood is donated either as whole blood or by using apheresis devices that extract one or more blood components and return the rest of the donation to the donor (4)

(Figure 1). After collection, whole blood is leukoreduced, mainly by filtration, in order to remove white blood cells, as well as clots and small clumps of platelets. Blood is then centrifuged in order to separate the different constituents (i.e., red blood cells, plasma and platelets). A biological qualification step is then performed; this consists of a series of biological tests, whose purpose is to detect viruses and bacteria circulating in the bloodstream. Plasma and platelets usually undergo a process of pathogen inactivation to reduce the risk of transmitting certain diseases by transfusion to the recipient patients (5). An example of one such process is the photochemical treatment of plasma with amotosalen and long-wavelength ultraviolet light (6); amotosalin is a synthetic psoralen, which intercalates between nucleic acids and, upon illumination with UVA light, forms covalent bonds preventing bacterial or viral replication (7); the plasma is then filtered from residual amotosalen and its degradation products. As an alternative to such processes, the plasma can be quarantined by keeping the plasma bag for a duration corresponding to "the window period" (at least 60 days for the French Blood Establishment (EFS)), while awaiting a second donation. During this silent period, a virus, even if present in the blood, may not be tested. When a second donation is made and its plasma tests negative for viruses and bacteria, the first donation is made available for transfusion.

Blood transfusions are generally considered safe, but evidence of certain harmful consequences has been reported (8); these can range from a mild allergic reaction to more

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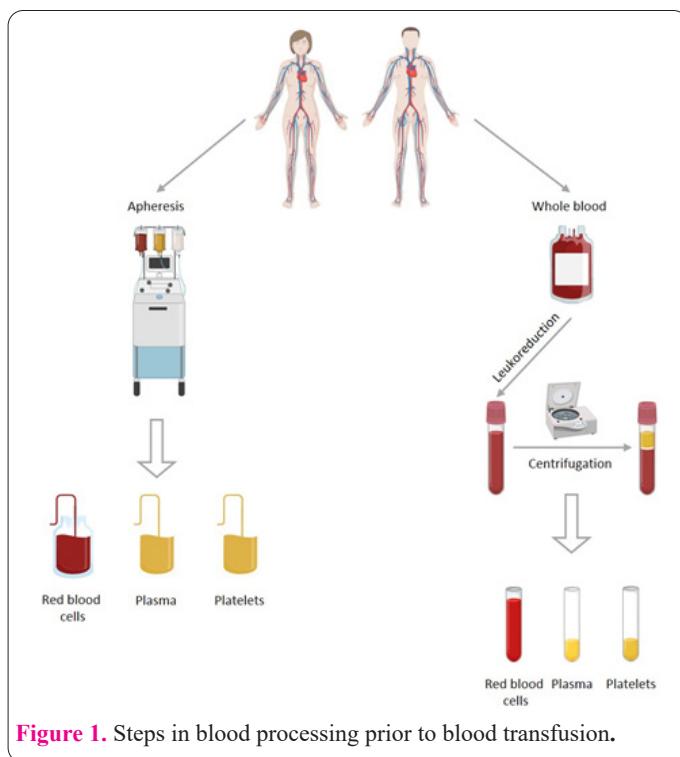


Figure 1. Steps in blood processing prior to blood transfusion.

serious effects, such as acute immune hemolytic reaction, in which the host immune system attacks the transfused red blood cells if the donor blood type is not a good match. However, it is the correlation between disease transmission and transfusion, that has caused the most concern (9). For that reason, the blood industry worked closely with the scientific community to improve and increase blood surveillance to the point where it was able to assert that blood was now “the safest it has ever been” (10). In fact, while transfusion guidelines take into consideration the age of blood components, the time when a patient is transfused, and other parameters (11), they take no account of other “structures” possibly present in the blood, such as cirDNA (12), extracellular vesicles, neutrophil extracellular traps (NETs) (13), or even circulating cell-free mitochondria (1) (Figure 2). The latter are macromolecule-based complexes, particles or cellular organelles, which we refer to as “biological structures” throughout the text below.

Circulating DNA

The presence of circulating extracellular DNA (or cell-free DNA, cirDNA) was first reported by Mandel and Mettais in the blood of healthy and cancer patients (14). It was then shown that cirDNA is also present in the blood of individuals suffering from autoimmune diseases, trauma, sepsis, myocardial infarction, and other diseases (2); and that DNA of fetal origin is found in the bloodstream of pregnant women (15). This DNA could be of nuclear or mitochondrial origin and circulates in the blood mainly in the form of mono-nucleosomes. Recently several reports revealed the high preponderance of mono-nucleosomes as being the most stabilized structures packing DNA. We recently demonstrated that about 90% of cirDNA are packaged in mono-nucleosomes (16). The remaining high molecular weight cirDNA is thought to be encapsulated in exosomes or microvesicles (2) or as a part of NETs.

The mechanism of cirDNA release has yet to be definitively confirmed but would appear to include cell death,

active secretion and other unknown mechanisms. Consequently, important considerations should be taken into account when transfusing patients. Studies have suggested that extracellular DNA, including mitochondrial DNA present in the extracellular milieu of transfused blood products, has biological actions capable of activating the innate immune system and inducing an inflammatory response, potentially contributing to some adverse reactions in transfusion (17) (Figure 3). This could be due to the double helix structure of the nuclear cirDNA and the particular motifs of certain sequences and molecular interactions (18,19). The exposition of macrophages to extracellular double-stranded DNA present in blood products eligible for transfusion increased the expression of genes involved in the innate immune response, including chemokines, chemokine receptors, and receptors of the innate response (20). In addition, levels of cirDNA were assessed in supernatants of stored red cell components as well as the effect of leukoreduction and gamma irradiation on the release of cirDNA during storage (21). It was shown that stored red cell components contain a significant amount of cirDNA and their release is further aggravated by irradiation while leukoreduction leads to a decrease in cirDNA content.

In physiological conditions, self-DNA released by dying cells is not detected by intracellular DNA sensors. Several works have revealed that excessive nucleic acids released from cells in interstitial space or in blood stream may re-enter the cell (22,23). This recognition is mediated by TLRs (Toll-like receptors) localized inside endosomes and lysosomes, and by cytoplasmic receptors such as retinoic acid-inducible gene 1 (22). Consequently, the re-entry capacity causes nucleic acids to be seen as “foreign invaders”, and may provoke a robust innate immune response (22). Interestingly, Poli et al (23) discovered that IL-26 binds to genomic DNA, mitochondrial DNA (mtDNA), and NETs, and shuttles them within the cytosol of human myeloid cells.

Since exacerbated inflammation has been associated with a failure in innate immune tolerance to self-DNA, in particular in inflammatory disorders, it is hypothesized that circulating DNA, mostly of mitochondrial origin, may be mediator of transfusion-related acute lung injury (TRA-LI) (24).

In addition to their immunological and proinflammatory role, cirDNA participate in horizontal gene transfer. In 1963, Stroun and Anker showed that a heterograft could cause changes that can be transmitted to the progeny in genetically stable plants (25). They hypothesized that these changes are due to a transfer of DNA from the mentor to the pupil plant and that this information is passed on to the progeny (26). In addition, it has been suggested that metastatic spread in cancer may be promoted by tumor DNA circulating in the bloodstream (27). Indeed, various studies have shown that fragments of cirDNA can penetrate into cells and modify their biology (28). In particular, the presence of cirDNA in the plasma of cancer patients has been shown to allow the transfer of oncogenes to cells in culture and to promote their transformation (29).

Simply by incubating NIH3T3 murine cells with the serum (or supernatant) of colon cancer patients, or in the culture medium of SW480 cells (human colorectal cancer cells), those cells can be transformed; Trejo-Becerril et al. postulated that this is due to the presence of extracellular

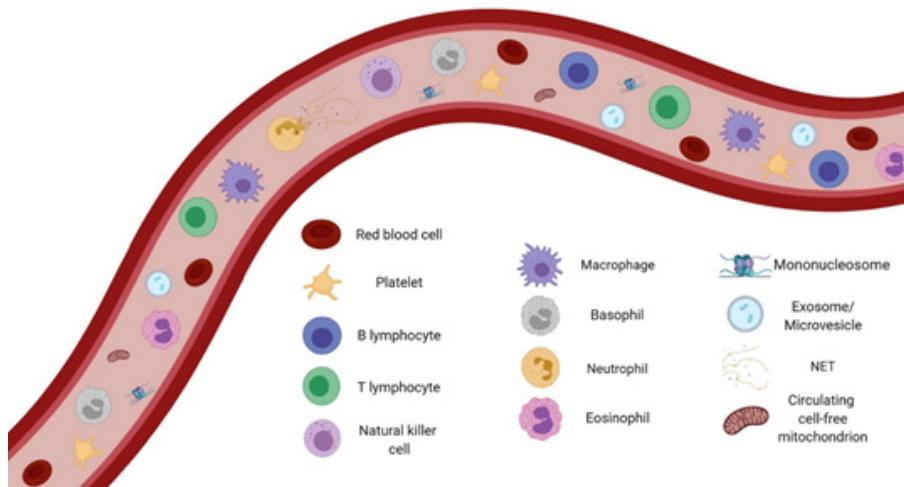


Figure 2. Different blood constituents.

DNA in that serum, supernatant or culture (30). García-Olmo et al. have proposed the theory of "genom metastasis" (31), according to which metastasis occurs by the transfection of sensitive cells located in distant target organs with dominant circulating tumor-derived oncogenes. This theory is consistent with the mechanism of horizontal gene transfer between different cells in the body (32,33). It has also been shown that blood transfusion can alter the hereditary traits of offspring in poultry (34). Whereas geno-metastasis might potentially have clinical incidence, one can consider that these transformed cells do not survive long time in the body, and this remains highly speculative.

Circulating mitochondria and circulating mitochondrial DNA

A recent breakthrough discovery demonstrated that blood contains circulating cell-free respiratory competent mitochondria (1), in addition to DNA of mitochondrial origin (mtDNA). Because of its bacterial antecedent, this DNA can serve as a DAMP (damage-associated molecular pattern) when released from cells (35) (Figure 3). In particular, mtDNA can bind to the TLR9 receptor of leukocytes via hypomethylated CpG motifs and trigger an inflammatory reaction (36). It was shown that interleukin 26 (IL-26), which is expressed in smooth muscle cells, can bind to genomic or mitochondrial extracellular DNA released from dying cells or [released] from NETs. The complex triggers the secretion of pro-inflammatory cytokines by monocytes, via the STING pathway and the inflammasome, to attract immune cells (23). Mitochondrial DNA-induced matrix metalloproteinase (MMP)-8/MMP-9 release by neutrophils, and intravenous injection of disrupted mitochondria (mitochondrial debris) into rats induced p38 MAPK activation and IL-6 and TNF- α accumulation in the liver (37).

Lee et al. demonstrated that mitochondrial DAMPs are present in red blood cells units, fresh frozen plasma, and platelets, and hypothesized that they might be mediators of TRALI (24), which is one of the most frequent and severe complications in patients receiving multiple blood transfusions. These observations were then confirmed, and it was shown that mtDNA DAMPs levels may predict the development of acute respiratory distress syndrome (ARDS) after multiple transfusions (38). Mitochondrial DAMPs also

potentiated inflammatory lung injury when introduced into healthy rats (39).

We recently found that platelet activation during standard plasma preparation led to the release of $\sim 376 \times 10^6$ full-length mtDNA copies per mL of plasma from healthy individuals, which approximately correspond to about 40 to 125×10^6 mitochondria per mL (40,41). Note, Al Amir Dache et al. (1) detected 200,000 to 4 million circulating cell-free mitochondria in blood using an advanced method to inhibit platelet activation during plasma preparation.

In addition to cell-free mitochondria (1,40), activated platelets were also found to release mitochondria encapsulated in extracellular vesicles (EVs) (43,44). Both entities are found in platelet concentrates used for transfusion, and are present at high levels in those that induced acute reac-

Structures	Reported potential detrimental effects
	- Activation of immune system - Induction of inflammatory response - Horizontal gene transfer - Genom metastasis
	- DAMPs: triggering the secretion of pro-inflammatory cytokines and attraction of immune cells, mediators of TRALI - Cell-free mitochondria: higher levels in transfused patients with acute reactions (febrile nonhemolytic reactions, skin manifestations, and cardiovascular events)
	- NETs' unbalanced formation and incidence in auto-immune diseases (vasculitis, obesity, Sickle Cell Disease, Cystic Fibrosis) - Toxic effects of NETs' by-products: tissue injury or inflammatory reaction - Attraction of cancer cells and formation of distant metastases
	- Carriers for intercellular communication - Immunomodulation and pro-inflammatory effect (e.g. autoimmune diseases) - Persistence and spread of virus - Neural dysfunction and mental disorders - Vascular endothelial dysfunction and promotion of cardiovascular diseases

Figure 3. Different structures present in the blood and their potentially detrimental effects; NETs: neutrophil extracellular traps; DAMP: damage-associated molecular pattern; TRALI: transfusion-related acute lung injury.

tions in transfused patients, such as febrile nonhemolytic reactions, skin manifestations, and cardiovascular events (42). Marcoux et al. have suggested that these platelet-derived EVs could be useful biomarkers for the prediction of potentially adverse transfusion reactions (45). In addition, it was recently shown that platelet-derived mitochondria are implicated in the up-regulation of naïve and central memory CD4+ T cells, the down-regulation of effector memory CD4+ T cells, and the modulation of cytokine production and gene expression (46). Boilard's group showed that platelets release mitochondrial antigens, especially cir-mtDNA, in systemic lupus erythematosus (SLE) causing acceleration and aggravation of the lupus phenotype, providing a rationale for the role of mtDNA in this auto-immune disease. Lastly, recent observations by Roch et al. (40) showed that detected cir-mtDNA in blood may mainly derive from intact mitochondria, suggesting a correspondence between detected cir-mtDNA and intact mitochondria number.

NETs

NETs are net-like structures composed of remodeled nuclear and mitochondrial chromatin, and containing antimicrobial granules of neutrophils (13). Their main function is to capture and eliminate pathogens. Excessive NETs formation may have serious physio-pathological effects. In fact, NETs dysregulation is associated with several autoimmune diseases (Figure 3) such as lupus or SLE (Systemic Lupus Erythematosus), arthritis, and diabetes, as well as with non-autoimmune diseases such as vasculitis, obesity, Sickle Cell Disease, or Cystic Fibrosis (47,48). In addition, serious toxic effects may occur when dysfunctional NETs formation produces by-products such as elastase or histones, resulting in endothelial or epithelial tissue injury or inflammatory reaction, respectively. Yang et al. recently showed that NETs act as a chemotactic factor to attract cancer cells and form distant metastases (49). NET-DNA is identified by the CCDC25 transmembrane receptor, which activates the ILK-β-parvin pathway to enhance cell motility. This study also reports that NETs are abundant in the liver metastases of patients with breast and colon cancer, and that they are predictors of such metastases in patients with early-stage breast cancer. Moreover, it was recently shown that activated platelets induce the formation of NETs in TRALI which can contribute to lung endothelial injury, and that targeting NET formation may be a promising new direction for the treatment of acute lung injury (50).

Extracellular vesicles: Exosomes and microvesicles

It has been reported that extracellular vesicles which are released into the circulation, such as exosomes and microvesicles, activate biological events and serve as carriers for intercellular communication (51,52) (Figure 3). These vesicles can be internalized by recipient cells, to which their contents, including DNA and RNA derived from the cell of origin, can be transferred.

Exosomes

Exosomes are a form of extracellular vesicles released by exocytosis, are 30-100 nm in size, and carry RNA and DNA fragments (53). A study has shown that exosomal

DNA represents the entire genome, and can reflect the mutational status of parental tumor cells in the case of cancer (54). Guescini et al. also reported that exosomes derived from astrocytes and glioblastoma cells can transport mtDNA (55). Exosomes have been identified as essential mediators for communication between cells through the transfer of proteins and genetic material, and have been reported to modulate the function of recipient cells, such as those involved in cancer, heart disease (51) and dysregulated inflammatory states such as sepsis (56).

It has been theorized that exosomes are the paracrine effectors of mesenchymal stromal cells (MSC). MSC-derived exosomes exert various biological functions, including multi-lineage differentiation, cytokines secretion, cellular proliferation and immunomodulation (57); this derives from the fact that immune cells for coordinated inflammatory responses, such as dendritic cells and T-lymphocytes, are able to absorb and secrete exosomes. It has also been shown that exosomes have the potential to transmit drug-specific hepatocyte-derived signals to the immune system and to provide a pathway for the induction of drug hapten-specific T-cell responses (58).

Mittelbrunn et al. reported exosome-mediated unidirectional transfer of miRNAs between T cells and antigen-presenting cells (59). Pegtel et al. demonstrated that exosome-mediated miRNAs secreted by Epstein-Barr virus (EBV) infected B cells were transferred to uninfected recipient cells (60). They also found that in peripheral blood mononuclear cells from patients with an increased EBV load, EBV miRNAs were present not only in B cells but also in uninfected non-B cells; this appears to suggest the transfer of miRNA via exosomes. DNA of the JC polyomavirus was detected in extracellular vesicles circulating in the plasma of healthy subjects, as well as in HIV-positive patients (61); this finding opens up new perspectives on the role of extracellular vesicles in the persistence of this virus, and in its spread to the central nervous system.

The role of exosomes was also studied in several autoimmune diseases (62). The presence of pro-inflammatory exosomes has been shown in the sera of SLE patients, where they induced the secretion of TNF-α and IFN-α in PBMCs, by way of a TLR-mediated mechanism (63). In addition, the exosomes' ability to cross the blood-brain barrier suggests that they might have a role in neural dysfunction and mental disorders (64). Studies have demonstrated that exosomes and their cargo play a role in communication within the central nervous system, as well as participating in nerve regeneration, synaptic function, plasticity, and immune response; they have also been implicated in the propagation of neurodegenerative diseases such as Parkinson's (65) and Alzheimer's (66).

Microvesicles

Microvesicles are another form of extracellular vesicles, range in size from 50 to 1000 nm, and are released into extracellular space by budding and fission of the plasma membrane (67). They carry and transfer genetic material to target cells, regulating their gene expression and protein synthesis, and affecting their biological function. Microvesicles can promote coagulation, induce angiogenesis, participate in immunomodulation, and initiate apoptosis after interactions with target cells (68). They mediate inflammation through the NF-κB signaling pathway (68). Additionally, they can enhance lung inflamma-

tion by carrying IL-1 β and TNF- α (69), and can increase the expression level of mRNA in receptor cells. Waldenström et al. showed that microvesicles derived from adult murine cardiomyocytes can transfer not only mRNA but also DNA to fibroblasts (70). Circulating microvesicles may transfer caspase-3 to target cells and regulate TNF- α and TRAIL signaling pathways, as well as cause cell death and promote the development of cardiovascular diseases (71). Other studies have shown that microvesicles containing microRNAs regulate the pathophysiological changes in vascular endothelium, such as cell apoptosis, proliferation, migration and inflammation, providing clues to their possible role in vascular endothelial dysfunction (72).

Conclusion

The implication of blood transfusion in various disorders is not only strongly suspected; in certain instances, as with TRALI, it is obvious, frequent and often severe. Given the high number of transfusions worldwide, however, the potential mediation of detrimental effects by transfusion deserves serious consideration. Notably, the effects of the recently discovered blood constituents we have described deserve investigation. That said, the need to perform such studies on a large cohort population is an obstacle to the achievement of that goal. Ideally, a large epidemiology correlation study between donor and host would be performed, in order to study the features of donors with diseases discovered immediately after blood transfusion; and to study hosts with chronic diseases associated with the release of one or more of these components.

In light of all of the above, it seems reasonable to suppose that transfusions have either adverse or favorable effects, given that the body's physiology rarely remains absolutely neutral in the presence of endogenous biological entities. This is particularly true when considering circulating factors of systemic potential, notably in the context of cell-to-cell communication. We, therefore, speculate that in particular conditions these circulating biological structures may have toxic effects, or may be used as messengers between organs or cells. Although extremely rare disorders do occur following blood transfusion, it might be hypothesized that these nanometer-scale structures cause detrimental physiological effects in individuals bearing host genetic factors which are difficult to identify. In this context, host acute and chronic clinical conditions should be flagged where transfusions are carried out, as a precautionary measure to be pointed out. For example, one can think that a non-diagnosed, asymptomatic cancer individual with a high level of cfDNA or NETs could donate blood which could subsequently cause an inflammatory response in the recipient. However, the question of tumor transmission or other diseases from a blood donor to a patient has always been a concern, and extensive research has addressed this question in animal models and follow-up of patients receiving blood from donors subsequently diagnosed with malignancy or other diseases, but the evidence is lacking to date for any connection.

Our discussion remains highly speculative, while blood transfusion remains one of the safest and most successful medical interventions in public health. The side effects which may result from these structures deserve to be investigated, to accompany the ongoing advances being made in characterizing these novel blood components.

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Interest Conflict

The authors declare no conflict of interest.

Author's contribution

RT and ART designed, and wrote the manuscript. Illustrations were made by RT on BioRender.

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