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Hypothesis

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# New insights into the possible role of bacteriophages in host defense and disease

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## Abstract

**Background:** While the ability of bacteriophages to kill bacteria is well known and has been used in some centers to combat antibiotics – resistant infections, our knowledge about phage interactions with mammalian cells is very limited and phages have been believed to have no intrinsic tropism for those cells.

**Presentation of the hypothesis:** At least some phages (e.g., T4 coliphage) express Lys-Arg-Gly (KGD) sequence which binds β3 integrins (primarily αIIbβ3). Therefore, phages could bind β3+ cells (platelets, monocytes, some lymphocytes and some neoplastic cells) and downregulate activities of those cells by inhibiting integrin functions.

**Testing the hypothesis:** Binding of KGD+ phages to β3 integrin+ cells may be detected using standard techniques involving phage – mediated bacterial lysis and plaque formation. Furthermore, the binding may be visualized by electron microscopy and fluorescence using labelled phages. Binding specificity can be confirmed with the aid of specific blocking peptides and monoclonal antibodies. In vivo effects of phage – cell interactions may be assessed by examining the possible biological effects of β3 blockade (e.g., anti-metastatic activity).

**Implication of the hypothesis:** If, indeed, phages can modify functions of β3+ cells (platelets, monocytes, lymphocytes, cancer cells) they could be important biological response modifiers regulating migration and activities of those cells. Such novel understanding of their role could open novel perspectives in their potential use in treatment of cardiovascular and autoimmune disease, graft rejection and cancer.

## Background

Bacterial predators, i.e. bacteriophages (phages) – viruses that infect and rapidly destroy bacteria, were discovered almost a century ago and there have been many attempts to apply phages in treating bacterial infections. While phage treatment has been successfully used in Russia, Georgia and Poland, it has been largely ignored in the West. The emerging crisis of antibiotic resistance and the

uncertain outlook for new antibiotics have dramatically altered this state of affairs, generating renewed interest in phages as a means of eradicating drug-resistant pathogens. Recently, *Science* published a comprehensive overview of the current situation and perspectives of phage therapy, highlighting the significance of our Institute's contributions [1].

While the ability of phages to attack bacteria has been known since their discovery (also reflected in their name), our knowledge about phage interactions with mammalian cells is very limited. In fact, phages have been believed to have no intrinsic tropism for those cells. Such tropism can be conferred on phages by fusing their surface proteins to a cell-targeting ligand. In this way, phage vectors have been adapted for targeted gene delivery [2]. On the other hand, phages are the most abundant life forms on Earth, being virtually omnipresent. Moreover, phage therapy is practically free of any side effects, as shown by results of phage treatment in the former Soviet Union and Poland (as well as in the USA, where phage preparations were licensed for sale in 1930s) [3,4]. They are consumed with foods, and some fresh water sources may contain up to $10^9$(billion) per ml [1,4]. Merril detected phages in animal sera and suggested that interactions between phages and mammalian cells should be investigated [5]. Furthermore, somatic coliphages can be detected in 68% and B.fragilis in 11% of the stools of healthy volunteers [6], whilst Enterococcus faecalis phages may be present in human saliva which suggest their role in the oral ecosystem [7].

Since phages are ubiquitous in our environment, the question arises whether human body can recognize them and mount an immune response. Indeed, elevated titers of antibodies against staphylococcal phage antigens can be detected in some 10% of healthy individuals and 44% patients with staph infections (in patients with acute infection, a 4-fold rise in antiphage antibody titer can occur). During regression of infection, a titer fall has been observed [8,9]. This data suggest that naturally occurring phages can induce humoral immunity. Antiphage antibodies can also be detected in the sera of patients treated with these phages [4,10]. Furthermore, *in vivo* humoral responses to phage phi X174 have been used for more than 30 years in clinical immunology as a measure of T-helper cell dependent antibody production [11–15]. *In vitro*, anti-phi X174 antibody is produced by lymphocytes from phage-immunized (but not unimmunized) subjects in the presence of antigen [16]. No data on potential T cell mediated anti-phage responses are available. These observations suggest that humans can mount at least a humoral response to phages and these appear to be innocuous (the phage used for immunocompetence testing could be repeatedly administered intravenously). The latter conclusion is also confirmed by the data of Wenger et al. who studied the *in vitro* effects of phages on human lymphocytes and embryonic kidney cells [17]. The authors found no viability or cytogenetic effects of phages in culture. It is also known that expression of eukariotic genes incorporated into the phage genome can take place as an effect of gene engineering (phages used as vectors for gene delivery). For example, phage lambda was used for complementation of the congenital absence of an enzyme in human fibroblasts [18]. Many investigations on this kind of engineered vectors have shown their ability of internalization into eukariotic cells but no observations of phage amplification or cell destruction have been described [19]. Therefore, it appears that phages are not detrimental to the host cells.

Can they bind to the host cells? As pointed out, both Lederberg and Merril were interested in a potential tropism of phages for mammalian cells and suggested that such interactions be investigated [5,20]. In fact, the preferential accumulation of phages in tumor tissues and inhibition of tumor growth was already demonstrated in the 1940s, and binding to neoplastic cells was confirmed later by Kañtoch et al. from our Institute [21,22]. They also suggested that T2 phages bind *in vivo* to guinea pig leukocytes (but not erythrocytes) [23]. In addition, Wenger et al. suggested that phages can attach to the plasma membrane of lymphocytes [17]. These interesting and potentially relevant observations were not pursued further, so the possible mechanisms responsible for the phenomena reported have not been unraveled.

Which receptors could be used by phages to bind mammalian cells and contribute to the described effects? It is known that viruses may utilize integrins (most often β3) for their attachment to cells (HIV, HPEV1 virus as well as hantavirus) [24–26]. Furthermore, adenovirus binding is also mediated by that integrin, and phages engineered so as to display the respective ligand can bind mammalian cells using β3 [27]. Nevertheless, phages could also use non-integrin receptors for binding to mammalian cells. For example, it has been suggested that CD26 is closely involved in HIV cell entry [28,29]. Furthermore, the non-integrin receptor DC-SIGN on dendritic cells may capture and transmit HIV to a wide variety of receptor-positive cells [30]. In addition, CMV envelope glycoprotein B is a viral ligand for that receptor [31]. The same receptor was shown to bind ebola glycoprotein and enhance infection of macrophages and endothelial cells [32]. Recently, viruses have been shown to interact with macrophages and lymphocytes and activate these cells using toll-like receptors [33,34]. Therefore, one could expect that naturally occurring phages may use similar mechanisms for possible interactions with host cells.

As mentioned earlier, there might also be other receptor:ligand systems enabling phage interactions with mammalian cells (both integrin and non-integrin). If they indeed exist, further genomic studies could unravel these potential mechanisms, which at the present time may only be speculative. Therefore, we believe that the proposed β3 : KGD binding interaction is currently the most plausible phenomenon suggesting that phage binding to the host cells may indeed occur.

## Presentation of the hypothesis

If phages indeed use a cellular receptor for their attachment to host cells, a suitable ligand for that receptor should be available on the phages. In fact, a KGD (Lys-Gly-Asp) sequence is present within the gp24 head corner protein of at least some phages (e.g., T4). There are 55 copies of this protein expressed by each phage, 5 at each head corner *(the complete genomic sequence of the T4 phage is available in databases* (see the National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/phg.html). According to these data, there is a KGD tripeptide sequence in a head corner protein. We confirmed this by a direct sequencing of gene 24 of our laboratory T4 strain (unpublished data).

This sequence is recognized by the major platelet integrin αIIbβ3 (the receptor for fibrinogen, von Willebrand factor, and some extracellular matrix proteins) [35]. The integrin may also be expressed by monocytes, some of which are β3+ and αvβ3+ [36]. Resting T cells are weakly β3+, but can acquire αIIbβ3 within as soon as 1 hr following *in vitro* activation (unpublished observations). Interestingly, it has recently been shown that this integrin may also be expressed by neoplastic cells, where it seems to be associated with their increased ability to grow and form metastasis [37,38]. Thus, our hypothesis implies that phages can bind host cells expressing αIIbβ3 and probably αvβ3 (an integrin having much lower affinity for KGD) [39].

Recently, a CD40 ligand (CD40L, CD154), known to activate endothelium and stimulate inflammation, was shown to have the KGD sequence, which allows it to bind to αIIbβ3 and activate platelets [39]. CD40L is crucial for T- and B-lymphocyte activation, and an interruption of CD40-CD40L interaction has been shown to inhibit graft rejection, autoimmune diseases, development of arteriosclerosis and angiogenesis [40,41]. KGD+ phages could compete with CD40L in its binding to β3 integrins on platelets and lymphocytes and prevent further activation of these cells. By binding αIIbβ3 on activated T cells these phages may coat their surface (phage opsonization), impair their ability to interact with adherent cells, endothelium and extracellular matrix proteins, and eventually cause their clearance from the circulation (similar to OKT3 monoclonal antibody and anti-lymphocyte globulin treatment).

The possible phage binding to platelets, monocytoid cells and lymphocytes may also explain why endogenous phages exert only a weak antibacterial action *in vivo*, whilst being so virulent against the same bacteria *in vitro*, a paradox that has never been adequately explained [1]. On the one hand, such binding could markedly reduce phage availability and dynamic interaction between phages and their bacterial targets *in vivo* (but not *in vitro*, where phages and bacteria confront each other directly, without the involvement of any other cellular interactions). On the other hand, the binding of phages to platelets, monocytoid and lymphoid cells may also explain why phages may be rapidly cleared from the blood by the spleen.

## Testing the hypothesis

### *In vitro*

Phages should bind cells expressing αIIbβ3 (and, to some extent, αvβ3), i.e. platelets, neoplastic cells (positive for β3), and activated T cells. This binding may be detected by the standard technique which detects plaque-forming units (PFU) produced by cell-bound phages lysing bacteria as originally used by Bloch [21] (which further indicates that different phage receptors are used for binding bacteria and mammalian cells) or by counting PFU formed by unbound phages present in cell-free supernatants. Furthermore, purified phages can be immobilized on plastic plates and cell adhesion may be evaluated using standard adhesion assays evaluated by ELISA. Binding may be confirmed by electron microscopy and fluorochrome-labeled phages, as recently described [42]. The binding specificity can be confirmed using agents known to block the function of β3 integrin (monoclonal antibodies as well as RGD- and KGD-containing peptides, e.g., eptifibatide (Integrilin), which contains a modified KGD sequence and is known to be a specific αIIβ3 blocker) [43]. In addition, platelet binding to fibrinogen may be impaired by phages containing the KGD sequence and thus competing with fibrinogen for αIIbβ3. Also, by a similar mechanism, phages should impair the binding of platelets to immobilized CD40L (competition in binding to platelet αIIbβ3 between the phage and the CD40L KGD sequence). Finally, the veracity of our hypothesis could also be confirmed by "the experiment of nature", that is, binding experiments using platelets from patients with Glanzmann's thrombastenia, an inherited platelet disorder characterized by a complete lack of platelet aggregation due to a defect in the αIIbβ3 complex or to a qualitative abnormality of this complex [44]; such platelets should not be able to bind KGD+ phages (in contrast to normal platelets).

***In vivo***

The administration of KGD+ phages may impair the growth and metastasis of tumor cells, which can be assayed using well-known systems in rodents (e.g., the effect on the formation of pulmonary metastases in mice by the β3+ B16 mouse melanoma cell line). In addition, platelet function can be assessed in phage-treated animals with the assumption that phage treatment could produce effects similar to those of platelet αIIbβ3 integrin blockers currently in use (e.g., eptifibatide).

## Implications

If, indeed, phages can bind mammalian cells and, perhaps, affect integrin β3 functions *in vivo*, this could revolutionize our current understanding of their biological significance. Consequently, they could no longer be considered solely as "bacteriophages" or bacterial viruses, as their anti-bacterial activity would only reflect one of their possible, multifaceted actions. Thus, such data would open completely new areas of study of the biological role of phages. Evidently, if phages can downregulate β3 integrin function, this could offer exciting and novel perspectives of their use, not only as antibacterial agents (whose efficacy could also be enhanced by blocking binding interactions), but also in the potential treatment of cardiovascular and autoimmune disease, cancer and transplant rejection. Phages could contribute to immunosurveillance against cancer by blocking β3 integrin activity on neoplastic cells thus preventing their growth *in situ* (induction of apoptosis?) and metastasis formation. Also, by occupying the αvβ3 integrin receptor, phages could deprive neoplastic cells from growth signals provided by extracellular matrix proteins [45]. Bloch demonstrated already in the 40th that injected phages store themselves electively in malignant tumors and that the Erlich carcinoma loses a high percentage of its transplantation ability upon phage addition [21]. Phage interactions with platelet β3 integrins and platelet-bound CD40L (which was recently shown to bind to platelets and stabilize arterial thrombi [39]), may produce effects similar to drugs interfering with their functions (e.g., eptifibatide) and thereby inhibit platelet hyperactivation which appears to play a prominent role in the initiation of atherosclerosis and its complications [46].

Of particular interest is the possible immunosuppressive effect of KGD+ phages which may coat activated T cells by binding to their αIIbβ3. Such phenomenon could be of importance in the digestive tract, where the immune system has to control the massive antigen challenge represented by the commensal bacterial flora. Some forms of experimental colitis in mice are dependent on hyperactivity of CD4+ T cells [47]. KGD+ phages could provide local immunosuppression and thereby prevent the development of autoimmune colitis. Phages occupying cellular receptors could prevent infection and re-infection of those cells (with their subsequent destruction) by pathogenic viruses using the same receptors for their cell entry. In other words, phages could engage in "a combat zone in the viral battlefield", as suggested by this type of reactivities at the level of plasma membrane and HIV infection [48] and thereby help our immune system that usually does not prevent the re-infection but does prevent clinical disease [49].

It is generally accepted that bacteria may be pathogenic or probiotic; the latter can generate immune signals which protect the human body against other pathogens [50,51]. This could be a more universal phenomenon and may also apply to what have been termed simply bacteriophages hitherto.

While further studies are needed to substantiate or dismiss these claims, confirmation of our hypothesis that phages bind to the mammalian cells would pave the way for exciting novel studies on the true place and role of phages in nature and their possible role in our defense against internal and external enemies.

## Competing interests

The Institute has filed a patent application covering therapeutic use of phages in bacterial infections.

## Authors contributions

AG conceived the hypothesis, designed the proposed experiments and drafted the manuscript; KD, KSJ and AO participated in conceiving the hypothesis, designing the experiments and preparation of the manuscript; BWD, JB, JW. and MN participated in designing the planned studies. All authors read and approved the final manuscript.

## Note

*Little is known of the interaction of bacteriophages with the mammalian host, even while they are universally present together with their bacterial hosts...*"

(J.Lederberg, Proc. Nat Acad Sci USA 1996, **93**:3167).

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