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Palaeobiology

Calcification of early vertebrate cartilage

Hagfish and lampreys are unusual for modern vertebrates in that they have no jaws and their skeletons are neither calcified nor strengthened by collagen — the cartilaginous elements of their endoskeleton are composed of huge, clumped chondrocytes (cartilage cells). We have discovered that the cartilage in a 370-million-year-old jawless fish, *Euphanerops longaevus*, was extensively calcified, even though its cellular organization was similar to the non-mineralized type found in lampreys. The calcification of this early lamprey-type cartilage differs from that seen in modern jawed vertebrates, and may represent a parallel evolutionary move towards a mineralized endoskeleton.

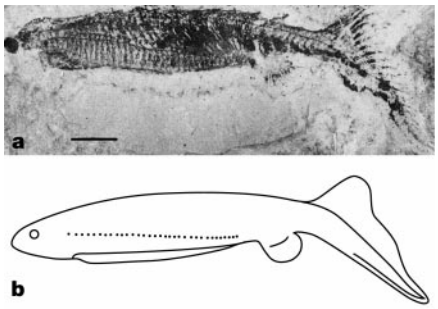

Figure 1 Young individual of the 370-million-year-old anaspid 'ostracoderm' *Euphanerops longaevus*. **a,** Specimen 2a from the Miguasha Museum of Natural History in Quebec, Canada. Scale bar, 10 mm. **b,** Reconstruction of the specimen in **a**. The specimen shows tarry imprints of cartilage that became calcified later in life.

E. longaevus is an anaspid 'ostracoderm' (one of an ensemble of jawless fish aged 470–370 Myr) from the Late Devonian locality of Miguasha, Canada (Fig. 1). Although the endoskeleton of anaspids was previously thought not to have been calcified, that of large, recently discovered specimens of *Euphanerops* shows extensive calcification. All endoskeletal elements of these specimens show the same, foam-like aspect and are made up of minute, hollow, spherical bodies that are loosely attached either by their walls or by an intervening matrix (Fig. 2a). Thin sections reveal that these spherical bodies are sometimes divided into two chambers, and that their walls are composed of calcified cartilage with evidence of growth rings.

This structure is strikingly similar to that of lamprey cartilage, in which large chondrocyte cells, often in groups of two or four (cell 'nests'[1]), are surrounded by a 'territorial' matrix with concentric rings that give rise to differential staining (Fig. 2b). In lamprey cartilage that is artificially calcified *in vitro*, calcium phosphate is deposited first in the territorial matrix (black dots in Fig. 2c) and then in the intervening matrix[2], generating the same pattern as is found in *Euphanerops*.

It is likely that the spherical bodies in *Euphanerops* contained chondrocytes, surrounded by calcified territorial matrix and held together loosely by a less-calcified intervening matrix. This process of calcification also occurs in the early stages of development of the higher jawed vertebrates (gnathostomes), but it must have occurred later in life in *Euphanerops*.

Ostracoderms are thought to be more closely related to gnathostomes than to either lampreys or hagfish[3,4], but our discovery suggests that the cartilage of *Euphanerops* was structurally similar to that of lampreys, and that it may also have been non-collagenous. This does not necessarily imply a close relationship between anaspids and lampreys, as has been proposed[5]. There is no evidence that the calcification pattern that is found in *Euphanerops*, and which can be imposed *in vitro* on lamprey cartilage, is a precursor of the large-scale, globular calcification of cartilage and bone that is seen in more derived ostracoderms and in gnathostomes, although it may represent a parallel — but less successful — move towards developing a calcified endoskeleton.

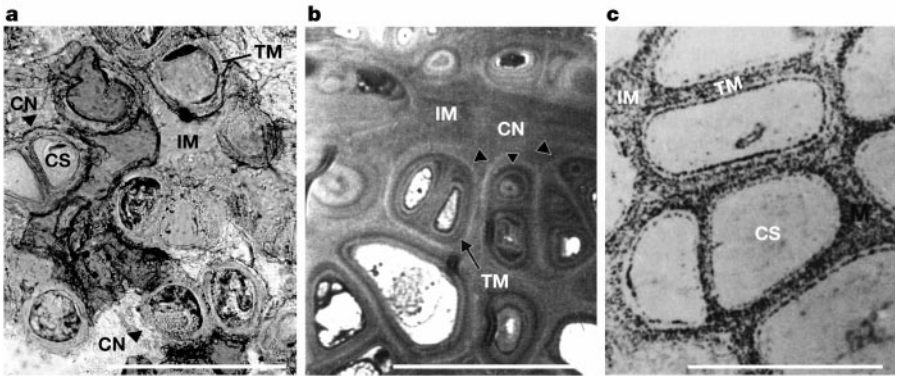

Figure 2 Micrographs of sections through an element of the vertebral column of a large specimen of *Euphanerops longaevus* (Miguasha Museum of Natural History no. 01-124) and through lamprey cartilage. **a,** *Euphanerops* displays large, rounded spaces for inclusion of chondrocytes, which are lined with calicified cartilage. **b,** Lamprey chondrocyte spaces in groups of two or four ('cell nests'), as in *Euphanerops*. **c,** Lamprey cartilage calcified *in vitro*, showing the same calcification pattern as in *Euphanerops*. Images in **b** and **c** are reproduced from ref. 4. CN, cell nest; CS, chondrocyte space; IM, intervening matrix; TM, territorial matrix. Scale bars, 50 μm.

Philippe Janvier*, Marius Arsenault†
**CNRS, UMR 8569, Muséum National d'Histoire Naturelle, 75231Paris Cedex 05, France*
e-mail: janvier@mnhn.fr
†Parc de Miguasha, CP 183, Nouvelle GOC2EO, Canada

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COMMUNICATIONS ARISING

Microbial evolution

Antitoxin vaccines and pathogen virulence

In their mathematical description of vaccine-induced immunity[1], Gandon *et al.* predict that the host's immunity to a microbial toxin will lead to increased virulence of the pathogen without affecting its transmission. However, results obtained using vaccines against two additional bacterial pathogens are not consistent with this prediction. The model proposed by Gandon *et al.* may therefore be an oversimplification, with the outcome depending on the biological details of both the pathogen and the vaccine.

The equation given by Gandon *et al.*[1] implies that antitoxin immunity always selects for higher virulence by reducing the risk of host death and hence selecting for more virulent variants. The authors conclude that antitoxin vaccines are therefore worse from an evolutionary and epidemiological viewpoint than vaccines that induce other types of immunity. They argue that virulence evolves with the efficacy of antitoxin vaccines because this type of vaccine removes the cost of virulence (increased mortality) without affecting its benefit for the pathogen (increased transmission).

Results obtained using diphtheria and

pertussis vaccines that induce antitoxin immunity and that are widely used in human populations argue against these predictions. The introduction of diphtheria toxoid vaccine at the beginning of the twentieth century led to a huge reduction in the number of people carrying the virulent form of this pathogen and to the persistence of non-virulent forms of the bacterium[2–4].

Diphtheria is caused by a toxin that is synthesized by *Corynebacterium diphtheriae*, which allows this bacterium to obtain nutrients when resources in the immediate vicinity are scarce. To produce the toxin, *C. diphtheriae* must carry a viral *tox* gene (*tox*$^+$ strain). Toxin production therefore confers a competitive advantage — cases of frank diphtheria are more contagious than cases of asymptomatic infection.

However, toxin production also carries a metabolic cost. As the toxin is neutralized in people who are immunized with diphtheria toxoid, its production is a drain on the bacterium, which is therefore at a competitive disadvantage. Accordingly, diphtheria has vanished from areas with long-standing and thorough diphtheria-toxoid vaccination programmes, whereas the *tox*$^-$ *C. diphtheriae* strain has persisted, a change that is attributable to the selective pressure exerted by the vaccine[5].

A similar mechanism could explain the impact of the pertussis-vaccination programme implemented in Sweden with a vaccine containing only pertussis toxoid, which also induces antitoxin immunity. This vaccine was introduced in 1995 in 11 Swedish counties to vaccinate all children between 6 months and 14 years of age. Four years later, the result of this programme was a large reduction in hospitalized pertussis cases, not only in vaccinated but also in non-vaccinated children (that is, infants younger than 6 months old and children older than 14 years). This demonstrates once again that antitoxin immunity does affect pathogen transmission[6–8].

Gandon *et al*. also argue that vaccines that counteract pathogen propagation may be less effective, as reduced transmission will elicit increased virulence. As we do not yet have an example of this type of vaccine for humans, we do not know whether this will be the case. This may be important for HIV vaccines[9] as well as for malaria, but we suspect that the reduction in transmission of a pathogen that replicates on mucosal surfaces will outweigh any possible increases in endogenous virulence.

Benoit Soubeyrand*, Stanley A. Plotkin†
**Department of Medical Affairs, Aventis Pasteur, 69007 Lyon, France*
†Department of Paediatrics, University of Pennsylvania, Pennsylvania 19104, and Aventis Pasteur, Doylestown, Pennsylvania 18901, USA
e-mail: stanley.plotkin@aventis.com

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Gandon et al. *reply* — Soubeyrand and Plotkin question our contention that antitoxin vaccines may select for greater pathogen virulence, arguing that this has not been borne out in real-life cases of diphtheria and pertussis, in which the widespread use of antitoxin vaccines has led to a reduced incidence of severe disease. They explain this success in terms of direct effects by the toxin on transmission that are both beneficial and costly. They argue that antitoxin vaccines have relieved the pathogen of the cost of high virulence due to host mortality (as we do too), but that these vaccines also maintain the metabolic cost of producing the toxin, helping natural selection to weed out the toxin producers.

In our model, we assume no such effects of toxin production — we envisage toxin production as an unavoidable, unhelpful side-effect of parasite replication, as seems to be the case in malaria. The apparent contradiction between our predictions and the observations cited by Soubeyrand and Plotkin is therefore due to differences in the life histories of different pathogens.

Our model can easily be extended to incorporate the costs and benefits of toxin production by modifying the pathogen's fitness function as follows:

$$R_0[\tau] = \frac{\beta[\alpha + (1-r)\tau]}{(\delta + \alpha + (1-r)\tau)} e^{-c\tau}$$

where τ is the level of toxin production, r is the efficacy of the antitoxin vaccine, $e^{-c\tau}$ is the cost function of toxin production, β represents parasite transmission as an increasing function of both toxin production and another component of disease-induced mortality, α, and δ is natural host mortality. Maximizing fitness yields the evolutionarily stable toxin production, τ^*, shown in Fig. 1. When the cost of toxin production is zero (as is assumed in our original model), virulence increases with vaccine efficacy. When the cost of toxin production is high, however, it counteracts the toxin's benefit to transmission, in which case optimal toxin production decreases with vaccine efficacy.

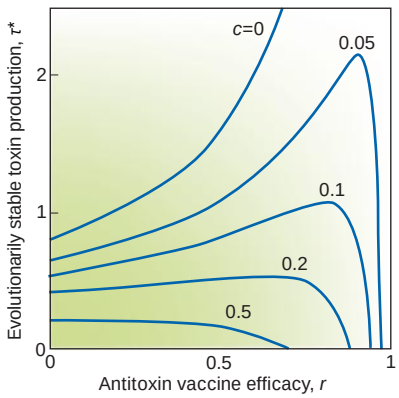

Figure 1 Evolutionarily stable toxin production, τ^*, plotted against antitoxin vaccine efficacy, r, for different toxin-production costs, c. Here it is assumed that all hosts are vaccinated, but similar results emerge for intermediate levels of vaccination coverage. The following transmission function was used: $\beta[\alpha+(1-r)\tau] = b_1(\alpha+(1-r)\tau)^{b_2}$. Parameter values: $b_1 = 1$, $b_2 = 0.5$, $\delta = 1$, $\alpha = 0.2$.

Figure 1 also shows that whereas highly effective antitoxin vaccines select for lower toxin production, imperfect vaccines can select for higher toxin production, which supports our argument that the use of imperfect vaccines can have negative consequences. The examples provided by Soubeyrand and Plotkin emphasize the need to understand how virulence and transmission relate to pathogen fitness for each disease of interest. Virulence evolution can occur in response to vaccination and other increases in host defence, both in positive ways, as Soubeyrand and Plotkin argue has occurred for diptheria and pertussis, and in negative ways, as others have argued may be the case in Marek's disease[1] and myxomatosis[2].

Sylvain Gandon, Margaret J. Mackinnon, Sean Nee, Andrew F. Read
Institute of Cell, Animal and Population Biology, University of Edinburgh, Edinburgh EH9 3JT, UK
e-mail: sylvain.gandon@ed.ac.uk

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