Reflections on filming living malaria parasites infecting red blood cells
By Geoff A. Butcher

The invention of the world wide web and developments in modern technology that enable the digitisation of cine film has provided an opportunity for those engaged in modern research to view images made many years ago. This is particularly relevant to those involved in malaria research who can now view a film made in 1969 which was the last section of my PhD **(1)**. These reflections are largely based on the PhD, with some additional observations. Further, the recent discovery that *P. knowlesi* can be added to the list of human malarias highlight its importance in research and therefore the value of the film.

The role of antibody in malarial immunity was first shown by Coggeshall and Kumn in 1937 **(2)** using the *P. knowlesi* rhesus model. In addition to being a non-human primate host, the rhesus is highly susceptible and can be infected with a single parasite. Infections are very synchronous and there is no immune response so that within a week or 10 days almost every red cell is infected. Following on from the work of Coggashall and Kumn, Cohen and McGregor further reinforced the protective effect of antibody by clearing infections of *P. falciparum* in children with immunoglobulin, from immune adults in the Gambia **(3)**.

With these experiments in mind, we hoped to use passive antibody transfer in *P. knowlesi* infected rhesus monkeys, to determine the effect of antibody on the blood stage of the life cycle - and from there to identify potential vaccine targets.

While we were able to show some protection in monkeys given a small infective parasite dose and immune IgG, this was not a practical method as it required a large dose of IgG to get significant protection **(4).** We therefore decided to use *in vitro* culture to determine the anti-parasite effectiveness of IgG from monkeys rendered immune by drug-controlled infections. We hoped that this would also lead to identification of the antibody target and thus to identifying protective antigens for a future vaccine against human malaria.

To the best of our knowledge this was the first attempt to visualise the interactions of the parasites and erythrocytes. The original film was shown only once at a meeting of the Royal Society of Hygiene and Tropical Medicine in 1970 **(5)** but is now available to a wider audience through the Welcome collection and the Royal Society of Tropical Medicine (https://wellcomecollection.org/works/sw476ssr). The film lasts 9 minutes but was the result of 24 hours of filming. It may be of interest to researchers studying red cells as well as immunologists and parasitologists.

The most significant observation from this work was the first observation that antibodies could inhibit merozoite invasion of red cells as previous suggested by Prof Cohen in the 1960s. Although in this film clear and rapid invasion of red cells by merozoites is not of the standard published 5 years later by **Dvorak** and colleagues **(6),** it does enable us to observe the different stages of this process. The focus of immunity appeared to be at the point of replication when the schizonts burst and a new cycle of invasion of red cells by merozoites occurs. In a meeting with Professors Cohen and Garnham and Dr Fulton, having reviewed my data, we decided this was the phase to be filmed.

Culture Methods and Observations

We began working with medium based on the Harvard Medium **(7)**. Samples of blood taken from monkeys with *P. knowlesi* were held at 37 ^{o}C under cover slips on the stage of the microscope and filmed at 16mm by phase contrast microscopy. At the time, the optimum culture conditions for

getting parasites through one growth cycle were still being developed. The first attempts to grow parasites in vitro failed, but after testing various components of the medium this was due to the normal monkey serum **(8)**. In failed cultures, it was noted that the red cells changed shape - varying from normal discoid to spherical form with minor projections. This condition is associated with patients who have a-beta-lipoprotein anaemia unable to transport vitamin E in the serum. The problem was solved mainly by improvements in the monkey diet with the inclusion of fresh fruit and vegetables. Interestingly, the addition of vitamins C and E direct to the culture medium had no beneficial effect, suggesting an indirect requirement.

While doing these experiments and attempting to improve parasite growth we tested sera from different animals and found that horse serum would support parasite growth and we used this in combination with the rhesus sera. This finding has proved useful in much later work when establishing *P. falciparum* and *P. knowlesi* in culture **(9);** and it is still widely used today for maintenance of P. knowlesi lines adapted to in vitro growth in human cells **(10)**.

In order to determine the events following the liberation of the merozoite from a bursting schizont, it was necessary to concentrate on a single schizont at a time. Eventually we realised that the schizonts burst only when the last pigment granule stuck to the residual body. The haemozoin particle was thus a useful morphological marker to predict progression to the next stage of the cycle - bursting of the schizonts. Whilst most merozoites appeared to explode out then undergo Brownian motion, in a few cases they streamed out in a long line through one hole in the schizont membrane.

On contact with the red cells the merozoites would make a loose attachment to the red cell surface and then fall away. If, however, this contact resulted in the apical end of the merozoite forming a junction with the red cell membrane the merozoite would then be internalised with the red cell, forming a parasitophorus vacuole. This has been described in many reviews at the electron microscope level **(11).**

The contact of red cells and merozoites results in quite dramatic alterations in shape, the red cell membrane appears to protrude and envelope the merozoite almost like an octopus enclosing its prey **(see Fig. 1)**. But in this case the tentacles may fall away. In this stage, now known as the "deformation", the contact between the red cell membrane and the merozoite is almost like a zip formation resulting from receptor-ligand interactions or possible differences in electrical charge.

Whatever other scientists may or may not learn from this old and relatively small contribution to malariology I hope they will find it useful and as fascinating as I did 50 years ago.

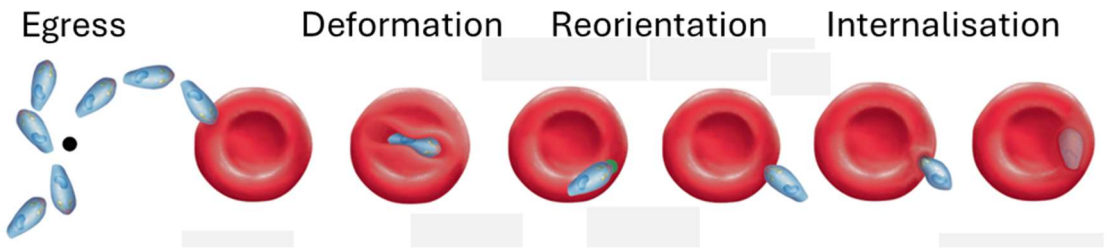

Figure 1: Schematic showing *P. knowlesi* invasion modified from **Hart *et al* 2023 (12)**. The merozoites are indicated in blue and the erythrocyte in red. Key stages shown here are visible in the videos – particularly the deformation stage where the red cell appears to wrap around the merozoite.

Acknowledgements and notes
I would like to thank Robert W. Moon for his advice and assistance in editing. For those interested in learning more about my life in research, my memoirs, entitled Memoirs of a Feeble Cabbage, are available from https://www.paperlionltd.com/clients/geoff-butcher-memoirs-of-a-feeble-cabbage/.

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