Letters

Malaria Diagnosis by Fluorescence Microscopy

Kawamoto and Billingsley describe a simple method for detecting haemoparasites stained with acridine orange (AO) using conventional light microscopes provided with adequate interference and barrier filters[1]. We have made a preliminary evaluation of this method. Both thick and thin smears, prepared with erythrocytes from *in vitro-Plasmodium falciparum* culture and blood from mice infected with *Trypanosoma cruzi*, were stained with an AO solution in 10 mM Tris–150 mM NaCl buffer, pH 7.5 (100 μg ml^{-1}). Examination was performed at 400× magnification under a Zeiss Axiophot microscope equipped with a 100W halogen bulb and filters kindly supplied by Dr Kawamoto. In all cases, parasites could be rapidly and easily identified.

Aiming at applying this method to malaria surveys in the Brazilian Amazon, where both *P. falciparum* and *P. vivax* infections are endemic, a slight modification of Kawamoto's thick smear technique was introduced. Approximately 5 μl of patient's blood were carefully mixed with dried AO on a microscope slide. A cover slip was added for examination. Slides with dried AO were previously prepared as follows: 10 μl of AO solution (100 μg of AO per ml 0.01 M phosphate-buffered saline, pH 7.2) were transferred to each slide and air dried; the slides were kept in the dark, at room temperature, until used. Otherwise, bacterial and/or fungal contamination could result from frequently pipetting AO solutions in field laboratories. Slides previously prepared with dried AO solution seem to be more convenient for use in malaria-endemic areas. However, further double-blind comparisons between results from examination of Giemsa- and AO-stained specimens are required before introducing this diagnostic technique into laboratory routine; this is the purpose of our current studies in the Brazilian Amazon.

Reference

1 Kawamoto, F. and Billingsley, P.F. (1992) *Parasitology Today* 8, 69–71

M.U. Ferreira and C.S. Ferreira
Department of Parasitology
Institute for Biomedical Sciences
University of São Paulo
Av. Prof. Lineu Prestes, 1374
BR-05508, São Paulo, SP Brazil

Reply

We are very pleased that Ferreira and Ferreira have modified the new 'thick smear' method from that described previously[1] for the acridine orange (AO) interference system (IFS). However, we are concerned that using dried AO on nonhaemolysed thick smears will cause unnecessary difficulty in detecting the parasites. Multilayered blood films physically impair observation of fluorescence emitted from stained parasites. This may be overcome if samples are haemolysed quickly by mixing with a one in ten volume of 1 mg ml^{-1} AO solution containing 1.5 M NH_4Cl or 0.01% saponin[2]. This method may result in an orange rather than red fluorescence. Even for very experienced workers in Thailand, the AO-IFS was at least as sensitive as Giemsa staining for thick smears (C. Wongsrichanalai *et al.*, unpublished). From some recent work, we have decided that thin smears are generally preferable for AO-IFS because: (1) thin smears are easier for inexperienced workers to simultaneously detect and identify parasites and (2) recent results from a blind trial in Sulawesi (Indonesia) comparing 513 thin smears indicated that the AO-IFS was an order of magnitude more sensitive than traditional Giemsa staining (16.2% versus 6.4% positives, respectively) (Syafruddin *et al.*, unpublished).

The AO-IFS requires strong light and, when the microscope is used with a mirror, the technique is dependent on weather conditions: it suffers from movement of the sun during the day and there are difficulties observing fluorescence in strong sunlight. We are well aware of these limitations. A slide projector (250W halogen or 1 kW tungsten bulb) can be used as a good alternative light source[2]. The AO-IFS has also been used to observe FITC-labelled immunofluorescence using natural and 'projector' illumination.

Many daylight microscopes have been replaced by tungsten-illuminated microscopes, and for these the AO-IFS works better with an optional mirror reflecting an external light source. In tungsten microscopes (20–30W), microfilariae have been optimally observed when the frosted glass filter was removed or with daylight microscopes illuminated by car headlights.

We are continuing to use, modify and improve the AO-IFS system, and we hope that, where other workers do the same, results will be published and forwarded to Dr Kawamoto as quickly as possible.

References

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F. Kawamoto
Department of Medical Zoology
Nagoya University School of Medicine
Nagoya 466, Japan

Syafruddin
Department of Parasitology
Toyama Medical and Pharmaceutical University
Toyama, Japan

C. Wongsrichanalai
Department of Immunology and Biochemistry
US Army Medical Corp
AFRIMS, Bangkok, Thailand

P.F. Billingsley
Department of Biology
Imperial College of Science, Technology and Medicine
London, UK SW7 2BB

Acquired Immunity in Schistosomiasis

Paul Hagan has convincingly reviewed the evidence for acquired immunity to (re)infection in human schistosomiasis[1]. We would like to make a few points that may be worth considering in future work:

(1) The conclusions of the reinfection studies in Kenya and The Gambia depend critically on the associated water contact studies. These have undoubtedly been carried out with great care, but details from either study have not yet been published. We would very much like to see this happen, if only for the sake of standardization of this tedious type of research.

(2) The assumption that the (cumulative) number of cercariae penetrating the skin during water contact is directly proportional to the number, duration and extent (body surface) of the contacts is not as obvious as may seem. A cluster of cercariae may infect a person whether he cools his feet for a minute or swims for an hour. In fact, we know little about either the dynamics or magnitude of the cercarial challenge to members of endemic communities.

(3) Another assumption is that individual worm loads are directly reflected by egg counts in urine or stools. The relationship between worm loads and egg counts demonstrated for *S. mansoni* is, in fact, far from linear or uniform[2], and *S. haematobium* egg counts must be interpreted with even greater caution[3]. Very little is known about the evolution of the relationship between worm loads and egg counts with age (of host and worm), after treatment and reinfection, or after development of immunity. Recent

work indicates, for example, that immunization may result in a strong decrease in worm fecundity[4], throwing an intriguing new light on the decline of egg counts in adults.

(4) The conclusion that acquired immunity is not concomitant, because it is effective in the absence of infection (presumably as determined by parasitological methods), is debatable. Repeated and/or more sensitive examinations, autopsies, serology and mathematical modelling show that most 'negative' people (also, and particularly, after treatment) in endemic areas carry light or unproductive infections[5–9], which may actually be a key factor in immunity.

(5) Mice exposed to natural waters may contract dozens of worms in a few hours[10,11]. Tourists reporting a single short exposure sometimes have egg counts comparable to those found in local communities, where children are intensively exposed, every day from early age. 'Slow' acquired immunity is certainly important, but surely there must be other, earlier forms of resistance that limit the number of (productive) worms in an individual and/or community. Another mystifying observation is that, in many areas, even of moderate endemicity, egg counts in children return to pretreatment levels (and no further) within one to two years of treatment[12–14], whereas it takes between five and ten years to build up the initial infection. At the individual level, we know of no model that explains or even addresses this phenomenon. However, it does suggest that there are still other mechanisms, largely independent of transmission, that regulate and truncate the parasite population in a community.

(6) It has been previously argued that schistosome populations must largely be regulated by host-related factors[15], rather than transmission[16]. Though both may play a role, it is now clear that host factors such as acquired (and other types of?) resistance are indeed major epidemiological determinants, particularly in areas with high (and probably 'excess'[15]) transmission. A practical consequence, which has not been considered so far, is that even successful efforts to reduce transmission may have little effect on the *outcome* of transmission, ie. the size of the parasite population. The surprisingly limited impact of snail control and chemotherapy on incidence, or on reinfection rates, observed in several control projects[7,12,14,17] may thus have a sound ecological basis.

Clustering of transmission may explain, but also complicate, some of these issues. In any case, we still know little about the transmission dynamics of schistosomes. As long as this gap persists, the basis for epidemiological and immunological modelling and for the development of transmission control or immunization strategies remains incomplete.

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Bruno Gryseels and Anton M. Polderman
Laboratory of Parasitology
University of Leiden
PO Box 9605, 2300 RC Leiden
The Netherlands

Reply

We agree with Gryseels and Polderman that the conclusions drawn from the reinfection studies are critically dependent upon the associated water contact studies. We will consider the points they raise one-by-one:

(1) Details of some of the water contact studies associated with the reinfection studies in The Gambia have been published[1,2] but we accept that further details should be more widely available. Several manuscripts based on this work are in preparation. Too often the importance of exposure to infection is understated. Like Gryseels, we are convinced that the most appropriate investments for transmission control are improved water supplies and sanitation (B. Gryseels, PhD thesis, University of Leiden, 1990).

(2) Given the paucity of hard data in the literature, we had to make some assumptions and feel that, in this case, our assumption that the (cumulative) number of cercariae penetrating the skin during water contact is directly proportional to the number, duration and extent (body surface) of the contacts is not an unreasonable one. However, we are aware of its limitations. We do not know, for example, whether an exposure of six minutes to water containing ten cercariae per litre is equivalent to an exposure of one hour to one cercaria per litre. Clearly the situation is complicated by the possibility that cercariae may be clumped, both spatially and temporally. In the absence of direct experimental evidence, this may be impossible to resolve.

(3) The relationship between individual worm loads and egg output in urine or stools may not be linear, uniform or indeed constant. The form of this relationship has been the subject of much debate. The evidence from human post-mortem studies[3] in which *Schistosoma mansoni* worms were recovered by perfusion and eggs in tissues and faeces were counted has been interpreted both in favour of[4], and against[5], density-dependent effects of worm load on egg output. Immunological factors may be even more significant through effects on worm fecundity. The evidence from vaccination studies on cattle exposed to *Schistosoma bovis* infection[6] strongly supports the idea of anti-fecundity effects of immunity. Recently, reductions in egg output have been reported in worms in mice immunized with the recombinant P28 (glutathione-S-transferase)[7] of *S. mansoni*. The pioneering work of the Leiden group in developing noninvasive assays for measuring worm burdens, as opposed to egg counts, should be immensely valuable in resolving this issue[8].

(4) We agree that repeated sampling and more sensitive assays are essential for accurate parasitological determinations. We routinely used triplicate noon urine specimens during the reinfection studies in The Gambia. Nevertheless, it is possible that some 'apparent negatives' may have been passing a very few eggs. It is equally possible that some people may have had unproductive infections. While light or unproductive infections may influence immunity, we and many others remain convinced that concomitant immunity is not the only form of immunity in schistosomiasis. Work on cattle and experimental animals using irradiated cercariae or antigen vaccines supports this view. Soon recombinant antigen vaccines against schistosomes will be available for testing in humans. Any immunity they stimulated would not be dependent upon concomitant immunity. This, of course, does not exclude the possibility that the actual effector mechanisms responsible for parasite attrition during concomitant immunity and during vaccine-induced immunity might be the same.

(5) On the question of the rapid reacquisition of infection, reinfection studies in The Gambia[1,9] showed that differences in levels of reinfection in young children were associated with differences in levels of exposure (as measured by an exposure index). In circumstances in which there is rapid reacquisition of infection by active 9-year-olds, this may partly reflect an increase in the levels of water contact over those they had as 6- or 7-year-olds. Density-dependent suppression of egg output may be a factor. Heavy pretreatment infection