Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 1167–1169, February 1999

Commentary

Malaria vaccines

*Anthony A. Holder**

Division of Parasitology, National Institute for Medical Research, Mill Hill, London NW7 1AA, United Kingdom

Malaria remains one of the most important vector-borne human diseases. Control of malaria caused by *Plasmodium falciparum* is a major goal, particularly for populations in sub-Saharan Africa. An effective vaccine against malaria that would protect nonimmune individuals from the disease has long been a dream. But will it be a reality? The concept that vaccination may be a useful tool to control the disease is based on a number of observations. Individuals continually exposed to infection by the parasitic protozoan responsible do eventually develop immunity to the disease, and passive immunization with antibody from immune donors can have a dramatic effect on blood stage parasitemia (1). Furthermore, inoculation of live attenuated parasites can protect naive volunteers against infection (2), and immunization with whole killed organisms can protect in animal models (3). Intensive studies of the immune response to the malaria parasite in humans and in models, particularly rodent malaria parasites in laboratory mice, have provided a wealth of information on potential protective mechanisms. Twenty years of antigen identification and gene cloning and expression, have produced many candidates for subunit vaccines (for a comprehensive recent review see ref. 4). Single proteins or peptides have been shown to be at least partially protective when used in vaccination studies in humans or in animal models (see, for example ref. 5). Within these antigens important epitopes have been identified, such as those recognized by neutralizing antibodies (6). All of these findings may suggest that an effective vaccine is "just around the corner," but what is reality, and what are the scientific obstacles?

By its very nature malaria vaccine research is a mix of empirical and rational approaches. It is hampered by the complexity of the parasite life cycle, imperfect tools to assess the efficacy of immune responses, and limited knowledge and understanding of the factors that determine the outcome of an infection. Optimism, fuelled by the desperate humanitarian need, often is tempered by the realization that we still know relatively little. Perhaps as part of a realistic assessment of the complexity of the interaction of the parasite and its host a multiplicity of approaches to vaccine development is being used. One approach is to identify individual elements of the parasite on which to focus the immune response, and then combine these elements into an immunogen that will prime the immune system to attack several different stages and targets in the parasite's life cycle. In the paper by Shi and colleagues (7), the approach taken is to "string together" as a synthetic gene sequences coding for peptides that form important epitopes. The recombinant protein expressed from this gene contains the "protective" epitopes from several proteins expressed at each stage of the parasite's life cycle. This multivalent, multistage approach is used to target multiple antigens expressed at different stages of the life cycle. Of particular interest is the fact that antibodies induced by immunization with this construct react with sporozoites, asexual liver, and blood stage parasites and gametocytes, and show activity in *in vitro* assays of function at two distinct stages of the parasite's life cycle. Unfortunately, it is unknown whether or not any of the available *in vitro* assays are predictive of functional immunity *in vivo*.

The life cycle of the malaria parasite is complex, the several stages in humans are morphologically and antigenically distinct, and immunity is stage specific. The genome of *P. falciparum,* which currently is being sequenced by The International Malaria Genome Sequencing Consortium (8, 9), probably contains about 5,000 genes, and only a subset of these is expressed at any one stage. It is only now becoming possible to define these patterns of expression and identify the full spectrum of proteins present at each stage.

Sporozoites are delivered by the bite of the infected mosquito, find their way to the liver, and invade hepatocytes. Just a handful of proteins expressed at this stage have been identified, and just two have been implicated in the recognition and invasion of hepatocytes. Antibodies to proteins on the parasite surface can neutralize sporozoites and prevent subsequent development of the liver stages.

Inside an hepatocyte the parasite differentiates and replicates asexually as a schizont to finally produce tens of thousands of merozoites that will initiate the blood stage of the infection. Antigens specific to the liver stage have been identified. It has been proposed that these antigens, together with those brought in with the invading sporozoite, are in part processed by the host cell and presented on the surface in combination with class 1 major histocompatibility complex. This presentation can lead to recognition by cytotoxic T-lymphocytes and killing of the infected cell, or stimulation of T cells to produce cytokines such as γ-interferon that can lead ultimately to the death of the intracellular parasite. Potentially any parasite protein expressed by the liver stage could stimulate the host immune system and lead to parasite killing at this stage.

Merozoites surviving immunity to the pre-erythocytic stages initiate the asexual blood stage infection, which is responsible for the disease. The parasite inhabits erythrocytes, and because these do not express major histocompatibility complex, parasite killing by cytotoxic T-lymphocytes is not important. Antibody binding to the surface of the merozoite and to proteins that are externalized from the apical complex of organelles involved in erythrocyte recognition and invasion probably plays a major role in immunity to asexual blood stages. Potentially this antibody could neutralize parasites, or lead to Fc-dependent mechanisms of parasite killing, for example, by macrophages. At least seven proteins associated with the merozoite surface and eight in the rhoptry and microneme apical organelles have been identified. In addition, expression of parasite proteins on the surface of the infected erythrocyte provides a target for antibody. Ligands involved in cytoadherence to endothelial cells and sequestration of the parasitized cell deep within tissues are expressed on the surface, although these proteins are encoded by a gene family and can undergo antigenic variation. The cyclic asexual mul-

The companion to this Commentary begins on page 1615.

*To whom reprint requests should be addressed at: Division of Parasitology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom. e-mail: aholder@nimr.mrc.ac.uk.

PNAS is available online at www.pnas.org.

tiplication in the blood stream provides a target to reduce parasitemia and thereby the severity of clinical disease.

Gametocytes also are formed in the blood and when ingested by a mosquito these sexual stages develop in the mosquito gut and initiate infection of the insect. Several proteins on the surface of the gametes/zygote have been identified as targets of antibody that blocks parasite development, thus transmission blocking immunity is the third target for the vaccine developers.

Because of the complexity of the parasite and its life cycle a multivalent multiple antigen vaccine is an attractive concept. It is based on what is probably a realistic assessment: that a single approach will never work 100% of the time. Enthusiasm for single antigen vaccines is tempered by a desire for a universally effective vaccine that would work in all circumstances, a vaccine that would be useful and acceptable for both the long-term inhabitants of malarious areas and for short-term nonimmune visitors. For public health programs protection of children and pregnant women from severe disease is a major concern. The idea is that while complete protection against initial infection with all parasite variants (and mediated by immunity to pre-erythrocytic stages) would be the outcome of an effective vaccine, a realistic assessment is that sterile immunity will not be achieved at all times. Therefore, a component that provides protection against the blood stage is an essential requirement to suppress parasitemia and reduce or abolish clinical disease. Following the same arguments, incomplete immunity to either pre-erythrocytic or asexual blood stages would allow sexual stages to be formed and transmission to the mosquito to continue. This outcome may be particularly worrisome if the partial immunity has selected resistant variants, which then would be selectively transmitted throughout the community.

The multivalent, multistage vaccine concept is not new but has not been fully evaluated so far. Hybrid proteins that contain sequences from a number of asexual blood stage antigens (10) or sporozoite and merozoite antigens have been analyzed (11–13), and immunization with a mixture of blood stage antigens has been attempted (14). A multiantigen approach to stimulate cytotoxic T-lymphocytes against liver stages also is being pursued (15), and a vaccinia virus NYVAC 7 containing seven genes has been evaluated in phase I/IIa clinical trials (16). Patarroyo *et al*. (17) developed SPf 66 as a synthetic peptide based on short sequences they identified as protective in a model infection, and this candidate vaccine has been tested, albeit with disappointing results, in field trials worldwide (18, 19).

The critical issues for the multivalent approach as with the single antigen approach are the identification of antigens at each stage that will induce a (partially) protective immune response in all or most of the target population, and the delivery of these antigens in a form that will stimulate the appropriate response. Selection of the antigen also must take into account mechanisms that the parasite has evolved to avoid effective immunity, for example, antigenic diversity, variation or more complex manipulation of the immune system such as stimulation of cross reactive low affinity antibodies (20), blocking antibodies that negate the action of neutralizing antibodies (21), or altered peptide ligand antagonism (22). The delivery system must allow presentation of the antigens in a form that stimulates the immune system. For recombinant or synthetic antigens this system almost certainly will require the development of adjuvants for human use that are more effective than those currently in other vaccines licensed for clinical use, although alternatives such as DNA vaccination or the use of viral vectors are being actively researched (23). Both aspects of identification and delivery critically depend on the definition and use of appropriate *in vitro* and *in vivo* assays that can predict efficacy of a vaccine in humans. There is still a long way to go to achieve this goal.

There are no *in vitro* assays of protective immunity that have been validated in vaccine trials. Despite this major impediment a number of assays have been used as surrogate markers for immunity and have been used to analyze immune responses of humans exposed to natural infection, as well as the outcome of experimental studies in humans and animal models. For example, the inhibition of sporozoite invasion (ISI) assay has been used to assess the ability of antibody to prevent parasite invasion of hepatocytes (24). This assay is technically demanding, requiring the production of viable sporozoites from infected mosquito salivary glands. A similar assay to assess the ability of antibodies to prevent parasite invasion of erythrocytes has been used extensively, and although easier to perform than the ISI assay no standardized and widely accepted format is used in the malaria research community (25). Druilhe and coworkers (26) have developed a variation on this blood stage assay they have called antibody-dependent cell inhibition (ADCI) in which addition of monocytes to the culture leads to differential parasite killing (26). A biochemical assay that measures the ability of antibody to inhibit the proteolytic processing of merozoite surface protein 1, which may be a key step in parasite entry into erythrocytes, also is being developed (21). Cellular responses measured by killing assays, proliferation, or cytokine production have been correlated with protection. Transmission blocking assays are conducted by membrane feeding mosquitoes with infected blood and counting the numbers of oocysts that develop on the gut wall.

Shi *et al.* (7) have produced a recombinant protein by expression of a synthetic gene that codes for short peptide sequences derived from two sporozoite proteins (CSP and SSP2), one liver stage-specific protein (LSA1), five asexual blood stage proteins (merozoite surface proteins, MSP1 and MSP2; rhoptry proteins, RAP1 and AMA1; and microneme protein, EBA 175), and one gametocyte protein (Pfg27). At first sight this would seem to be an unlikely vaccine candidate, because protective antibody directed against at least two of the antigens (MSP1 and AMA1) is thought to require disulfide bond-dependent conformational epitopes (27, 28). However, antibodies raised to this recombinant protein react with the recombinant protein itself, as well as with some of the constituent peptides in ELISA. Furthermore they react with sporozoites, liver and blood asexual stages, and gametocytes when assessed by immunoelectron microscopy, and with blood stages by immunofluorescence, indicating that they also react with the native proteins. The antibodies are active in the inhibition of sporozoite invasion and antibody-dependent cell inhibition assays (but do not affect asexual blood stage parasite growth in the absence of monocytes), and are inactive in transmission blocking assays.

How can this work be taken further? Clearly the antibody response of individual rabbits gives only a preliminary insight into how a human population might respond to the recombinant protein. The cellular responses to the antigen were not investigated and no assay of antiliver stage immunity was used. Proof of concept *in vivo* is also difficult to achieve without going straight to human studies because there are no good models for *P. falciparum* infection by mosquito challenge and blood stage development. What is the contribution of the response to the individual sequences to the overall response measured in the *in vitro* assays? Presumably the sequences that do not elicit a response are dispensable and could be replaced by other candidate sequences. Affinity selection of sequence-specific antibodies could be used to dissect in more detail the contribution of these antibodies to the effect measured in the *in vitro* assays.

In the near future the entire genome of *P. falciparum* will have been sequenced. This work undoubtedly will reveal many genes that code for proteins, as yet unknown, that may be good candidates for vaccine development. In a multistage, multiva-

lent vaccine strategy there will be almost limitless combinations of antigens or epitopes to analyze and assess. Even now there are not the resources or populations to take a purely empirical approach to this task. Thus if we are to develop an optimal vaccine against malaria it will have to be based in large part on an understanding of the important immune mechanisms involved in protection against the parasite and the disease (pathogenesis, immune evasion), the role of individual proteins in the parasite-host interaction, and the development of sensitive and meaningful *in vitro* methods to study these aspects.

1. Cohen, S., McGregor, I. A. & Carrington, S. C. (1961) *Nature (London)* **192,** 733–737.
2. Clyde, D. F., Most, H., McCarthy, V. C. & Vanderberg, J. P. (1973) *Am. J. Med. Sci.* **266,** 169–177.
3. Mitchell, G. H., Butcher, G. A. & Cohen, S. (1975) *Immunology* **29,** 397–407.
4. Hoffman, S. L., ed. (1996) *Malaria Vaccine Development: A Multi-Immune Response and Multi-Stage Perspective* (Am. Soc. Microbiol., Washington, DC).
5. Stoute, J. A., Kester, K. E., Krzych, U., Wellde, B. T., Hall, T., White, K., Glenn, G., Ockenhouse, C. F., Garcon, N., Schwenk, R., *et al*. (1988) *J. Infect. Dis.* **178,** 1139–1144.
6. Yoshida, N., Nussenzweig, R. S., Potocnjak, P., Nussenzweig, V. & Aikawa, M. (1980) *Science* **207,** 71–73.
7. Shi, Y. P., Hasnain, S. E., Sacci, J. B., Holloway, B. P., Fujioka, H., Kumar, N., Wohlhueter, R., Hoffman, S. L., Collins, W. E. & Lal, A. A. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 1615–1620.
8. Carucci, D. J., Gardner, M. J., Tettelin, H., Cummings, L. M., Smith, H. O., Adams, M. D., Venter, J. C. & Hoffman, S. L. (1998) *Curr. Opin. Infect. Dis.* **11,** 531–534.
9. Gardner, M. J., Tettelin, H., Carucci, D. J., Cummings, L. M., Aravind, L., Koonin, E. V., Shallom, S., Mason, T., Yu, K., Fujii, C., *et al*. (1998) *Science* **282,** 1126–1132.
10. Knapp, B., Hundt, E., Enders, B. & Kupper, H. A. (1992) *Infect. Immun.* **60,** 2397–2401.
11. Holder, A. A., Lockyer, M. J. & Hardy, G. W. (1988) *Parasitology* **97,** 373–382.
12. Lockyer, M. J., Cooper, H., Tite, J., Rowan, W. & Crowe, J. S. (1993) *Parasitology* **106,** 451–457.
13. Sturchler, D., Just, M., Berger, R., Reber-Liske, R., Matile, H., Etlinger, H., Takacs, B., Rudin, C. & Fernex, M. (1992) *Trop. Geog. Med.* **44,** 9–14.
14. Sturchler, D., Berger, R., Rudin, C., Just, M., Saul, A., Rzepczyk, C., Brown, G., Anders, R., Coppel, R., Woodrow, G., *et al.* (1995) *Am. J. Trop. Med. Hyg.* **53**, 423–431.
15. Gilbert, S. C., Plebanski, M., Harris, S. J., Allsopp, C. E. M., Thomas, R., Layton, G. T. & Hill, A. V. (1997) *Nat. Biotech.* **15,** 1280–1284.
16. Ockenhouse, C. F., Sun, P.-F., Lanar, D. E., Wellde, B. T., Hall, B. T., Kester, K., Stoute, J. A., Magill, A., Krzych, U., Farley, L., *et al*. (1998) *J. Infect. Dis.* **177,** 1664–1673.
17. Patarroyo, M. E., Amador, R., Clavijo, P., Moreno, A., Guzman, F., Romero, P., Tascon, R., Franco, A., Murillo, L. A., Ponton, G. & Trujillo, G. (1988) *Nature (London)* **332,** 158–161.
18. D'Alessandro, U., Leach, A., Drakeley, C. J., Bennett, S., Olaleye, B. O., Fegan, G. W., Jawara, M., Langerock, P., George, M. O., Targett, G. A. & Greenwood, B. M. (1995) *Lancet* **346,** 462–467.
19. Nosten, F., Luxemburger, C., Kyle, D. E., Ballou, W. R., Wittes, J., Wah, E., Chongsuphajaisiddhi, T., Gordon, D. M., White, N. J., Sadoff, J. C., *et al*. (1996) *Lancet* **348,** 701–707.
20. Anders, R. F. (1986) *Parasite Immunol.* **8,** 529–539.
21. Guevara Patiño, J. A., Holder, A. A., McBride, J. S. & Blackman, M. J. (1997) *J. Exp. Med.* **186,** 1689–1699.
22. Plebanski, M., Lee, E. A. & Hill, A. V. (1997) *Parasitology* **115,** Suppl.**,** S55–S66.
23. Wang, R., Doolan, D. L., Le, T. P., Hedstrom, R. C., Coonan, K. M., Charoenvit, Y., Jones, T. R., Hobart, P., Margalith, M., Ng, J., *et al*. (1998) *Science* **282,** 476–480.
24. Hollingdale, M. R., Ballou, W. R., Aley, S. B., Young, J. F., Pancake, S., Miller, L. H. & Hockmeyer, W. T. (1987) *Exp. Parasitol.* **63,** 345–351.
25. Chulay, J. D., Haynes, J. D. & Diggs, C. L. (1981) *J. Infect. Dis.* **144,** 270–278.
26. Bouharoun-Tayoun, H., Attanath, P., Sabchareon, A., Chongsuphajaisiddhi, T. & Druilhe, P. (1990) *J. Exp. Med.* **172,** 1633–1641.
27. Chappel, J. A. & Holder, A. A. (1993) *Mol. Biochem. Parasitol.* **60,** 303–312.
28. Hodder, A. N., Crewther, P. E., Matthew, M. L., Reid, G. E., Moritz, R. L., Simpson, R. J. & Anders, R. F. (1996) *J. Biol. Chem.* **271,** 29446–29452.