Plasmodium knowlesi Sporozoite Antigen: Expression by Infectious Recombinant Vaccinia Virus

Abstract. *The gene coding for the circumsporozoite antigen of the malaria parasite* Plasmodium knowlesi *was inserted into the vaccinia virus genome under the control of a defined vaccinia virus promoter. Cells infected with the recombinant virus synthesized polypeptides of 53,000 to 56,000 daltons that reacted with monoclonal antibody against the repeating epitope of the malaria protein. Furthermore, rabbits vaccinated with the recombinant virus produced antibodies that bound specifically to sporozoites. These data provide evidence for expression of a cloned malaria gene in mammalian cells and illustrate the potential of vaccinia virus recombinants as live malaria vaccines.*

Malaria remains a serious global health problem for which there is no effective vaccine. Protective immunity against malaria infection has been obtained following inoculation of animals with irradiated sporozoites (*1*). The use of monoclonal antibodies indicated that the surface protein is the target for neutralization of infectivity (*2, 3*). Recently, the gene encoding the circumsporozoite protein of *Plasmodium knowlesi* has been cloned and the immunogenic region identified as a tandemly repeated epitope (*4–6*). Candidate subunit vaccines might be prepared either by engineering the genes encoding such antigens into prokaryotic or eukaryotic expression vectors or by chemical synthesis of an immunogenic peptide. An alternative approach is to use vaccinia virus as a vector (*7, 8*) for the construction of a live recombinant virus that would be administered in the same manner as smallpox vaccine. Thus far, infectious vaccinia virus recombinants expressing hepatitis B virus surface antigen (*9–11*), influenza virus hemagglutinin (*12, 13*), and herpes simplex virus glycoprotein D (*11*) have been shown to induce appropriate antibody production in vaccinated animals. Moreover, a single intradermal vaccination protected animals against hepatitis B (*14*) or influenza virus (*12*) and an intraperitoneal injection provided protection against herpes simplex virus (*11*). Here we extend these studies by describing vaccinia virus recombinants that express the surface antigen of a malaria parasite.

The expression of foreign genes in vaccinia virus was facilitated by construction of a series of specially designed plasmid insertion vectors (*15, 16*). These contain a defined vaccinia virus promoter upstream from unique restriction endonuclease sites and flanked by vaccinia virus DNA from a nonessential region of the virus genome. Foreign genes can be placed under the control of the vaccinia virus promoter and inserted into infectious virus by homologous recombination in vivo. By inserting the foreign gene into the thymidine kinase (TK) locus (*17, 18*), recombinants display a selectable TK^- phenotype.

A 1.6-kilobase fragment, obtained by cleavage with the restriction enzyme Aha III and originally cloned from blood stage DNA of *P. knowlesi* (*5*), was inserted into plasmid insertion vector pGS20 (*15, 16*). This fragment contains the entire coding sequence for the circumspor-

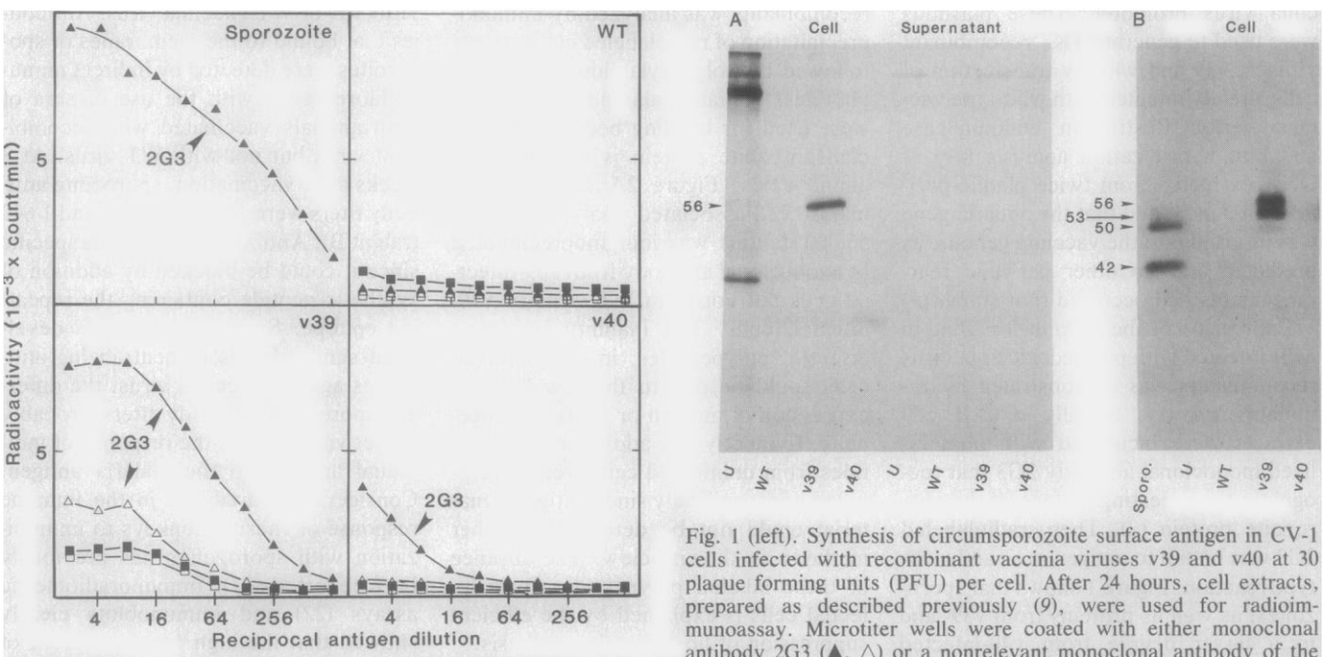

Fig. 1 (left). Synthesis of circumsporozoite surface antigen in CV-1 cells infected with recombinant vaccinia viruses v39 and v40 at 30 plaque forming units (PFU) per cell. After 24 hours, cell extracts, prepared as described previously (*9*), were used for radioimmunoassay. Microtiter wells were coated with either monoclonal antibody 2G3 (▲, △) or a nonrelevant monoclonal antibody of the same isotype (■, □), and then incubated with serial dilutions of cell extracts (WT, v39, and v40) or sporozoites (initial concentration 5×10^7 per milliliter). After the unbound material was washed away, the wells were incubated with ^{125}I-labeled monoclonal antibody 2G3 in the presence (△, □) or absence (▲, ■) of 5 μ*M* Gln-Ala-Gln-Gly-Asp-Gly-Ala-Asn-Ala-Gly-Glu-Pro. Unbound material was washed away and the ^{125}I remaining in the microtiter wells was counted. Fig. 2 (right). Malaria polypeptides synthesized in recombinant vaccinia virus–infected cells. (A) Immunoprecipitation. CV-1 cells were infected with WT vaccinia virus or recombinant v39 or v40 at 30 PFU per cell or were mock-infected (U). After 1 hour the inoculum was removed and the cells were incubated in medium lacking serum for 1 hour and then in the same medium supplemented with [^{3}H]alanine and [^{3}H]glycine (100 μCi per cell monolayer) for 3.5 hours. Equivalent fractions of cell extract or culture supernatant were incubated with monoclonal antibody 2G3 for 16 hours at 4°C. Immune complexes were precipitated with Formalin-fixed staphylococcal A protein, washed as described previously (*9*), resolved by electrophoresis through a 15 percent polyacrylamide gel, and autoradiographed. (B) Immunoblotting. Extracts of CV-1 cells infected with WT vaccinia virus or recombinant v39 or v40 were dissociated with sodium dodecyl sulfate and mercaptoethanol and resolved by electrophoresis through a 7.5 percent polyacrylamide gel. Solubilized extracts of the salivary glands of mosquitos infected with *P. knowlesi* were run in parallel (lane Sporo.). Separated proteins were transferred to nitrocellulose by electroblotting (*24*) and incubated successively with monoclonal antibody 2G3 for 12 hours and ^{125}I-labeled affinity purified rabbit antibody to mouse IgG for 8 hours at 20°C. An autoradiograph is shown with indicated molecular weights ($\times 10^{-3}$) for the polypeptides.

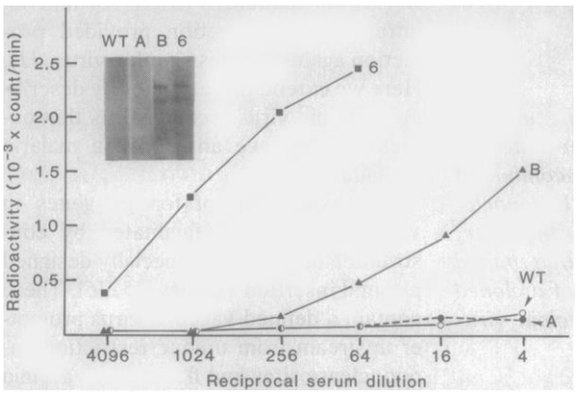

Fig. 3. Detection of sporozoite antibody in the serum of vaccinated rabbits. Microtiter wells were coated with extracts of *P. knowlesi* sporozoites as described (*2*). Rabbit sera obtained 3 weeks after a single intradermal inoculation of 10^8 PFU of vaccinia virus (rabbit WT) or recombinant v39 (rabbits A and B), or hyperimmune sera from a rabbit immunized with 10^6 to 10^7 *P. knowlesi* sporozoites in complete Freund's adjuvant and boosted by intravenous injections (rabbit 6), were serially diluted and added to the wells. After incubation at room temperature for 2 hours and washing, ^{125}I-labeled affinity-purified goat antibody to rabbit IgG was added. After 1 hour, unbound material was washed away and the wells were cut and counted. The inset shows the results of an immunoblotting experiment. Circumsporozoite antigens from *P. knowlesi*–infected mosquito salivary glands were resolved by polyacrylamide gel electrophoresis and transferred to nitrocellulose. The membranes were incubated for 2 hours with the diluted rabbit sera, then washed and incubated with ^{125}I-labeled affinity-purified goat antibody to rabbit IgG prior to autoradiography.

ozoite protein. The resulting plasmid, pGS39, contains a vaccinia virus transcriptional start site 310 bp upstream from the only translational initiation codon in frame with the repeating epitope of the malaria gene. A control plasmid, pGS40, contains the malaria gene incorrectly oriented with respect to the vaccinia virus promoter. These plasmids were used to generate TK$^-$ recombinant viruses, v39 and v40, by transfection of cells already infected with wild-type vaccinia virus. Restriction endonuclease and blot hybridization analysis (*19*) of DNA extracted from twice plaque-purified virus indicated that the malaria gene was inserted into the vaccinia genome as predicted and no other genomic rearrangements had occurred (not shown).

Expression of the *P. knowlesi* gene in cells infected with purified vaccinia virus recombinants was demonstrated by radioimmunoassay. Serially diluted cell extracts were incubated with immobilized monoclonal antibody 2G3 that recognizes the repeating epitope of the sporozoite protein (*2*). Then, radiolabeled 2G3 was bound to antigen in a sandwich-type reaction. Figure 1 shows that sporozoites, as well as proteins from v39- and to a lesser degree from v40-infected cells, reacted with monoclonal antibody 2G3. In contrast, proteins from cells infected with wild-type (WT) vaccinia virus or uninfected cells (not shown) did not. The specificity of this reaction was demonstrated by blocking antibody binding with chemically synthesized peptide (*20*) representing the repeating epitope of the malaria protein (*6*). The 30-fold lower level of expression of immunoreactive material in v40-infected relative to v39-infected cells is similar to that obtained when other foreign genes were inserted into vaccinia virus without a properly oriented vaccinia virus promoter (*9, 16*). Additional controls indicated that infected cell extracts did not react with an unrelated monoclonal antibody.

The sporozoite polypeptide synthesized in cells infected with vaccinia virus recombinants was analyzed by immunoprecipitation of radiolabeled cell extracts followed by polyacrylamide gel electrophoresis. Tritiated alanine and glycine were used for labeling because the circumsporozoite protein is rich in these amino acids. Figure 2A shows that a major cell-associated polypeptide of 56,000 daltons was immunoprecipitated by monoclonal antibody from v39-infected cells but not from WT virus or v40-infected cells. The inability to detect discrete polypeptides in v40-infected cells could be due to the low level of expression of antigen or to its heterogeneity. Evidently, the additional polypeptides from uninfected cells were precipitated nonspecifically since antigenic material could not be detected by other methods as shown below. The absence of these labeled polypeptides from infected cells is explained by the efficient suppression of host protein synthesis.

For a direct comparison, the proteins synthesized in cells infected with vaccinia virus recombinants were analyzed on the same polyacrylamide gel as proteins from the salivary glands of *P. knowlesi*–infected mosquitos (Fig. 2B). After transfer to nitrocellulose, circumsporozoite antigens were detected by binding of monoclonal antibody 2G3 followed by binding of ^{125}I-labeled affinity purified rabbit antibody to mouse immunoglobulin G (IgG). In v39-infected cells two major polypeptides of 56,000 and 53,000 daltons were produced, whereas polypeptides of 52,000, 50,000, and 42,000 daltons were obtained from infected mosquito salivary glands. The latter is thought to be the fully processed form found on the surface of sporozoites (*2*). The differences in mobility of recombinant virus and sporozoite-derived polypeptides are probably not due to glycosylation since there are no consensus glycosylation sites. Moreover, there was no detectable incorporation of ^{3}H-labeled glucosamine into the recombinant virus–derived polypeptide (not shown). Utilization of different translational initiation codons seems unlikely since no new in-frame ATG triplet (A, adenine; T, thymine; G, guanine) was introduced during the construction of the chimeric gene. Other possibilities include differences in proteolytic processing of the sporozoite polypeptide in the virus-infected mammalian cell or the use of different translational termination codons.

To test the immunogenicity of the polypeptide synthesized by the vaccinia recombinant, we vaccinated rabbits intradermally with purified recombinant virus v39 or WT vaccinia virus. Antibodies that bound to the membranes of sporozoites were detected by indirect immunofluorescence with the use of sera of both animals vaccinated with recombinant virus but not with WT virus. At 3 weeks after vaccination, sporozoite antibody titers were 1/80 (rabbit A) and 1/640 (rabbit B). Antibody binding was specific since it could be blocked by addition of synthetic peptide containing the repeating epitope. Since both animals developed similar levels of neutralizing antibodies against vaccinia virus, the different sporozoite antibody titers probably reflect variations in the response of individual animals to the malaria antigen. Considerable variability in the immune response of rhesus monkeys to immunization with sporozoites has previously been reported (*21*). Immunoradiometric assays (*22*) and immunoblots clearly showed that the high titer antiserum of rabbit B reacted with the mature and precursor circumsporozoite proteins (Fig. 3). As a positive control, the same figure shows the results of titration and blot analyses of the serum of an animal (rabbit 6) repeatedly immunized with sporozoites.

An understanding of the structure and function of the surface antigens of malaria parasites will undoubtedly help in the development of vaccines. Here we report what appears to be the first expres-

sion of a cloned malaria gene in a eukaryotic vector. The sporozoite surface antigen synthesized by the infectious vaccinia virus recombinant reacted with monoclonal antibody specific for the repeating epitope of the malaria protein. Furthermore, intradermal inoculation of rabbits with infectious recombinant virus stimulated specific antibody production. Significant increases in the synthesis of the sporozoite antigen are to be expected with additional genetic engineering. On the basis of the large capacity of vaccinia virus for foreign DNA (*23*), it should be possible to express several antigens from different life stages of the malaria parasite simultaneously to create a more potent vaccine. By incorporating genes of additional infectious agents that pose health problems in areas of the world where malaria is endemic, polyvalent vaccines may be produced.

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25. We thank N. P. Salzman and T. McCutchan for critical reading of the manuscript.

27 February 1984; accepted 20 March 1984

Repression of Rearranged μ Gene and Translocated c-*myc* in Mouse 3T3 Cells × Burkitt Lymphoma Cell Hybrids

Abstract. *The productively rearranged immunoglobulin μ chain gene and the translocated cellular oncogene c-*myc *are transcribed at high levels both in human Burkitt lymphoma cells carrying the t(8;14) chromosome translocation and in mouse plasmacytoma × Burkitt lymphoma cell hybrids. In the experiments reported here these genes were found to be repressed in mouse 3T3 fibroblast × Burkitt lymphoma cell hybrids. Such repression probably occurs at the transcriptional level since no human μ- and c-*myc *messenger RNA's are detectable in hybrid clones carrying the corresponding genes. It is therefore concluded that the ability to express these genes requires a differential B cell environment. The results suggest that the 3T3 cell assay may not be suitable to detect oncogenes directly involved in human B cell oncogenesis, since 3T3 cells apparently are incapable of transcribing an oncogene that is highly active in malignant B cells with specific chromosomal translocations.*

In Burkitt lymphoma cells with the t(8;14) chromosome translocation, the c-*myc* oncogene, normally located on band q24 of chromosome 8, translocates to the immunoglobulin heavy chain locus on chromosome 14 (*1–3*). By contrast, in Burkitt lymphoma cells with either the t(8;22) or the t(2;8) variant chromosomal translocation, the chromosomal breakpoint is distal (3′) to the c-*myc* oncogene and either the immunoglobulin λ locus or the immunoglobulin κ locus, respectively, translocates to a DNA region distal (3′) to the untranslocated and unrearranged c-*myc* oncogene (*4–5*). As a result of its close proximity to rearranged

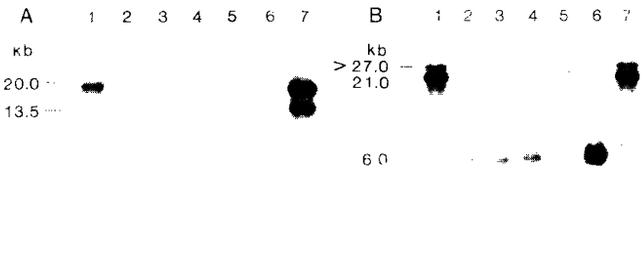

Fig. 1. Southern blotting analysis of hybrid cell DNA for (A) human μ genes and (B) c-*myc* genes. Agarose gel (0.7 or 1 percent) electrophoresis was carried out in 40 m*M* tris-HCl, 5 m*M* sodium acetate, and 2.0 m*M* EDTA, *p*H 8.0. Hind III digested phage λ DNA (0.75 μg per lane) size markers were included in every gel. Cellular DNA samples were digested with restriction enzymes and then subjected to electrophoresis in a horizontal agarose gel (10 μg of DNA per lane). Transfer of DNA from gel to nitrocellulose sheet was performed essentially as described by Southern (*14*). The DNA probes were labeled by the nick translation procedure (*21*). The DNA on nitrocellulose sheets was hybridized to ^{32}P-labeled probe DNA in a hybridization solution containing 50 percent (by volume) formamide. (A) Southern blotting analysis of hybrid cell DNA after Bam HI digestion and with the use of a 1.2-kb Eco RI genomic DNA probe of the Cμ gene (*2*). This probe includes the first, the second, and part of the third exon of the Cμ gene (*2*). Lane 1, JD38 lymphoma cell DNA; lanes 2 and 3, IT × JD-A and IT × JD-B hybrid DNA's, respectively; lane 4, IT22 mouse parental DNA; lanes 5 and 6, hybrid 272-3 and 272-5 DNA's, respectively; lane 7, ST486 Burkitt lymphoma DNA. The two IT22 × JD38 hybrid (272-3 and 272-5) DNA's (lanes 2 and 3) have the rearranged 20.0-kb band that contains the expressed μ gene. Both of the ST486 × IT22 hybrids (lanes 5 and 6) contain the rearranged but unexpressed Cμ gene (*9*) while only hybrid 272-3 contains the productively rearranged Cμ gene (13.5 kb) (*9*). (B) Southern blotting analysis of hybrid cell DNA's following Bam HI digestion and using a human c-*myc* cDNA probe (Rye 7.4) containing sequences derived from the second and third exon of c-*myc* (*3*). Lane 1, JD38 lymphoma DNA; lanes 2 and 3, hybrid IT × JD-A and IT × JD-B DNA's, respectively; lane 4, IT22 mouse parental DNA; lanes 5 and 6, hybrid 272-5 and 272-3 DNA's, respectively; and lane 7, ST486 Burkitt lymphoma cell DNA.