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Anchoring a Secreted Plasmodium Antigen on the Surface of Recombinant Vaccinia Virus-Infected Cells Increases Its Immunogenicity

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We show that the subcellular location of foreign antigens expressed in recombinant vaccinia viruses influences their effectiveness as immunogens. Live recombinant viruses induced very poor antibody responses to a secreted repetitive plasmodial antigen (the S-antigen) in rabbits and mice. The poor response accords with epidemiological data suggesting that S-antigens are poorly immunogenic. Appending the transmembrane domain of a membrane immunoglobulin (immunoglobulin G1) to its carboxy terminus produced a hybrid S-antigen that was no longer secreted but was located on the surface of virus-infected cells. This recombinant virus elicited high antibody titers to the S-antigen. This approach will facilitate the use of live virus delivery systems to immunize against a wide range of foreign nonsurface antigens.

Since the development of methods for the expression of foreign genes in infectious vaccinia virus (10, 15), live recombinant vaccinia viruses have been shown to be of great potential in immunizing animals against infection with other pathogenic viruses. This has been achieved by isolating the genes for antigens that are targets of host-protective immune responses and integrating them, under the control of vaccinia virus promotor elements, into the vaccinia virus genome. The foreign viral antigen expressed by the vaccinia virus is processed, modified, transported to the infected-cell surface, and presented to the host immune system in a manner very similar to that in a normal infection. It is therefore not surprising that when the herpes simplex glycoprotein D (5, 16), hepatitis B surface antigen (13, 18), vesicular stomatitis virus glycoprotein G (12), and influenza virus hemagglutinin (14, 19) genes were inserted into vaccinia viruses, these live recombinant viruses effectively immunized animals against the relevant infections.

Effective immunization with recombinant vaccinia viruses may depend on the foreign antigen being expressed on the surface of the virus-infected cell. However, many antigens which are important in the development of host-protective immune responses to more complex intracellular pathogens, such as malaria parasites, may not be transported to the cell surface when the relevant genes are introduced into mammalian cells. For example, the *Plasmodium falciparum* proteins which are located in the knob structures on the surface of mature parasite-infected erythrocytes and involved in the adherence of the infected erythrocyte to the vascular endothelium must be transported from the parasite and across the parasitophorous vacuole membrane and erythrocyte cytoplasm before reaching their final destination. Other antigens such as the ring-infected erythrocyte surface antigen accumulate in the apical organelles of the developing merozoite and are transferred to the surface of newly infected erythrocytes during the invasion process. Expression of such genes in recombinant vaccinia viruses may not be expected to generate cell surface molecules.

Another problem in effective immunization may relate to the repetitive sequences which are a dominant characteristic of many of the antigens of *P. falciparum*. It has been suggested that they prevent the development of high-affinity responses (1) and thus may hold the key to understanding the molecular basis of immune evasion in this host-parasite relationship.

In this report we focus on a secreted repetitive protein of *P. falciparum*, the S-antigen of the Papua New Guinea isolate FC27. S-antigen is secreted by the parasite into the parasitophorous vacuole and is released from this vacuole into the bloodstream during rupture of the mature schizont. The sequence of S-antigen genes from two different isolates of *P. falciparum* (4) indicates the presence of a short region at the 5′ end of the gene which would code for a hydrophobic signal peptide but no other significant regions of hydrophobicity in the rest of the gene, consistent with this characterization as a secreted protein. These antigens of unknown function are highly variable among isolates of *P. falciparum*, giving rise to in excess of 50 serotypes of this protein. The serological data also suggest that S-antigens are not very immunogenic. The processes by which such diversity in the S-antigens is generated remain obscure, although it is difficult to implicate the involvement of selective immune pressure on this class of poorly immunogenic, secreted molecules.

We show here that the S-antigen of the FC27 isolate is secreted from cells infected with the recombinant vaccinia virus expressing this protein. However, in rabbits and mice immunized with the infectious recombinant virus, the immune response to the S-antigen protein was negligible. Retargeting the S-antigen to the surface of infected cells by adding a membrane anchor domain resulted in a dramatic increase in its immunogenicity.

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MATERIALS AND METHODS

Plasmid constructions. An 880-base-pair (bp) *Aha*III fragment containing a deleted version of the FC27 S-antigen gene was isolated from the genomic *Eco*RI clone FC27.4.S described by Cowman et al. (4). *Eco*RI linkers were added before cloning this fragment into pUC9. This 880-bp subclone (pFC27Aha2) encoded an almost complete copy of the S-antigen including the 23-amino acid hydrophobic signal sequence, the 68-amino acid conserved amino terminus, 13 copies of the 11-amino acid repeating peptide of which we estimate there are 100 copies in the undeleted protein, and the complete 35-amino acid sequence of the conserved carboxy-terminal end of the molecule. As well there were 40 and 35 bp of 5′ and 3′ noncoding flanking DNA, respectively. The 888-bp *Eco*RI fragment was then cloned into the single *Eco*RI cloning site of the vaccinia transfection vector pGS62 which was constructed by deleting one of the *Eco*RI restriction enzyme sites in the vector pGS20 described by Mackett et al. (11). Recombinants were selected in which the S-antigen gene was inserted in the correct orientation 3′ to the vaccinia 7,500-molecular-weight protein (7.5K protein) early-gene promotor and flanked on either side by the 5′ and 3′ ends of the vaccinia virus thymidine kinase (TK) gene sequences of the plasmid vector. This new construct (pV8) was used to transfect CV-1 cells infected with wild-type vaccinia virus, giving rise to the recombinant vaccinia virus V8 containing the S-antigen gene.

Addition of mouse membrane immunoglobulin transmembrane sequence to S-antigen gene. A 186-bp *Hae*III fragment containing sequences encoding 6 amino acids of the hinge region, 26 amino acids of the transmembrane domain, and 28 amino acids of the intracellular domain of mouse immunoglobulin G1 (IgG1) was isolated from the γ1 cDNA clone described by Tyler et al. (20). *Sph*I linker DNA with the sequence 5′-GCGCATGCGC-3′ was then ligated to the *Hae*III fragment which was then digested with *Sph*I and cloned into the unique *Sph*I site located 65 bp from the 3′ end of the S-antigen gene in the subclone pFC27Aha2.

The resultant clone pA20 containing the inserted *Sph*I fragment in the correct orientation with respect to the S-antigen gene was then digested with *Eco*RI, and the 1,084-bp fragment was cloned in the correct orientation into the *Eco*RI site of the pGS62 vector described above to yield the plasmid pVA20. This plasmid DNA was used to transfect vaccinia-infected CV-1 cells to produce the recombinant vaccinia virus VA20.

Methods for production and selection of recombinant vaccinia virus. Methods for the production and selection of recombinant vaccinia virus were as described by Mackett et al. (11) with the exception that single virus plaques were selected by two rounds of endpoint dilution in 96-well microtiter trays containing monolayers of human 143B TK^- cells in the presence of 25 μg of 5-bromodeoxyuridine per ml. Recombinant viruses containing the S-antigen genes were screened for the presence of DNA by dot-blot analysis or for the production of S-antigen which was detected by a high-titer polyclonal antiserum, R210, raised by immunizing rabbits with a β-galactosidase-fused polypeptide from clone Ag16 (3) containing 23 copies of the 11-amino acid FC27 S-antigen repeating polypeptide.

Expression of S-antigen in recombinant vaccinia-infected cells. Confluent monolayers of BSC-1 cells were routinely infected at 1 PFU per cell with purified recombinant virus and allowed to incubate at 37°C for 18 to 48 h. At this time the infected cells and the supernatant were harvested and dissolved in sodium dodecyl sulfate (SDS) sample buffer and boiled. Samples were then analyzed by immunoblotting and probed with a rabbit anti-S-antigen antiserum, R210, which recognizes the repeating epitope of the FC27 S-antigen molecule.

Triton X-114 partitioning. Recombinant vaccinia-infected cells were dissolved in 0.5% Triton X-114 in phosphate-buffered saline (PBS) for 1 h at 4°C. After centrifugation at 2,000 rpm to remove nuclei, the Triton X-114-soluble material was layered over a cushion of 0.06% Triton in 6% sucrose–PBS, and then the temperature was raised to 37°C. The cloudy suspension was separated by centrifugation at 37°C. The oily Triton X-114 pellet should contain the integral membrane proteins by virtue of the greater affinity of their hydrophobic transmembrane sequences for the Triton X-114 detergent which becomes insoluble at elevated temperature (2). The supernatant, which should contain soluble proteins, was also collected. Each fraction was subjected to a further cycle of purification to reduce contamination. Samples of each fraction were then added to SDS sample buffer, separated by polyacrylamide gel electrophoresis, and analyzed by immunoblotting.

Immunofluorescence. BSC-1 cells were grown onto sterile glass cover slips for a period of 6 h after which they were infected at approximately 0.5 PFU per cell with either the V8 or VA20 recombinant virus or the TK^- nonrecombinant virus as a control. After 18 h, cover slips were rinsed in cold PBS and then stained immediately with rabbit R210 anti-S-antigen antiserum followed by fluorescein isothiocyanate-conjugated sheep antirabbit antibodies. Cells were then postfixed in cold 95% ethanol–5% glacial acetic acid before mounting under glycerol and visualization by indirect fluorescence microscopy. A parallel group of infected cells was fixed in cold 95% ethanol–5% glacial acetic acid before staining to permeabilize the cells.

Immunization of animals. Rabbits were immunized with a single intradermal injection of 10^8 PFU of purified recombinant or TK^- nonrecombinant virus on their lower back followed by a second immunization 7 weeks later. Lesions appeared beneath the skin within a few days of the first immunization, reaching a size of approximately 1 to 1.5 cm in diameter. Occasionally these lesions ulcerated. Lesions were no longer apparent after 4 weeks. Rabbits were bled at weekly intervals, and the sera were analyzed for anti-S-antigen or antivaccinia antibodies in an enzyme-linked immunosorbent assay (ELISA). Female age-matched inbred mice of various strains were immunized with a single intraperitoneal injection of 10^7 PFU of virus followed by a second challenge by the same route 3 weeks later. Sera were collected 3 weeks after the primary immunization and 12 days and 3 weeks after the rechallenge. Anti-S-antigen and antivaccinia antibody titers were assayed by serial dilution of the sera in an ELISA. Poor antivaccinia antibody titers were induced in male mice immunized intraperitoneally with 10^7 PFU of recombinant vaccinia virus. Therefore, only female mice were used in this study.

ELISA determination of antibody responses. Antibodies to the repeat portion of the FC27 S-antigen protein were assayed in an ELISA in which 96-well polyvinylchloride microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated overnight at 4°C with the purified Ag16 fusion polypeptide at a concentration of 5 μg/ml in PBS. Antivaccinia antibody titers were determined with plates coated with a TK^- nonrecombinant virus preparation which had been inactivated by treatment with β-propiolactone. Sera were diluted in PBS containing 0.5% bovine serum

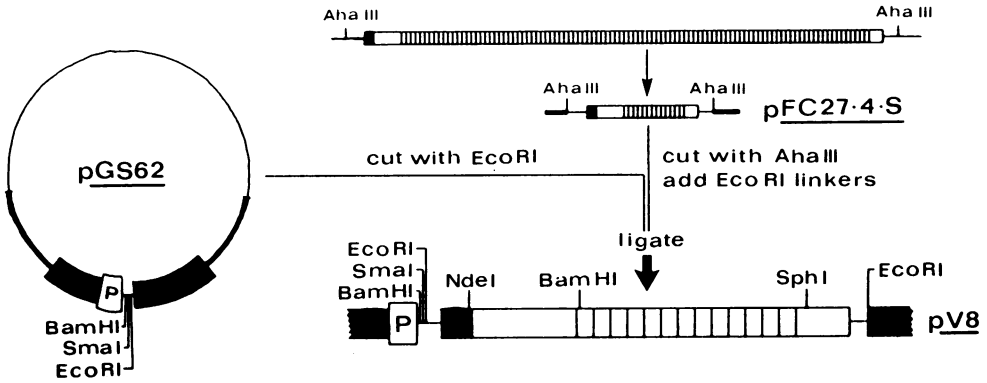

FIG. 1. Construction of the transfection plasmid pV8 containing the deleted S-antigen gene of the FC27 isolate of *P. falciparum*. The structure of the FC27 S-antigen gene with sequences encoding a signal peptide (dark shading) and approximately 100 copies of the 33-bp repeating sequence is shown at top right. Cloning of this gene and accompanying deletion of sequences in the repetitive region of the gene yielded the 3.7-kilobase genomic subclone FC27.4.S described by Cowman et al. (4) containing all nonrepeat sequences of the S-antigen gene but only 13 copies of the repeat sequence. This DNA was cleaved at the *Aha*III restriction endonuclease sites 40 bp 5′ and 35 bp 3′ to the coding region of the gene. After the addition of *Eco*RI linkers, this fragment was cloned into the unique *Eco*RI restriction site of pGS62 (see Materials and Methods) to yield the plasmid pV8. In this construct the S-antigen gene is located immediately downstream from the vaccinia virus 7.5K gene promotor (P) and is flanked on both sides by vaccinia virus TK gene sequences shown in dark shading.

albumin and 0.05% Tween 20, and 50-μl samples were incubated in the antigen-coated wells for 2 h. As the secondary antibody, horseradish peroxidase conjugated to sheep anti-rabbit immunoglobulin or sheep anti-mouse immunoglobulin was used at a dilution of 1:1,000. Bound conjugate was determined from the absorbance value at 415 nm 1 h after the addition of the substrate 2,2′-azinobis-3-ethylbenzthiazoline sulfonic acid.

RESULTS

Expression of the FC27 S-antigen gene of *P. falciparum* in recombinant vaccinia virus. The S-antigen gene of the FC27 isolate of *P. falciparum* was cloned into the *Eco*RI site of the vaccinia virus transfection plasmid pGS62 (a derivative of pGS20 described by Mackett et al. [11]) as described in the Materials and Methods (Fig. 1). In this construct (pV8), the initiation codon for the S-antigen gene lies 40 bp downstream from the *Eco*RI cloning site which is adjacent to the vaccinia virus 7.5K gene promotor. Initiation of translation at this methionine codon and termination at a TGA codon 822 nucleotides downstream results in a protein of 274 amino acids in length or approximately 28 kilodaltons in size after cleavage of the signal peptide. This protein contains 13 copies of the 11-amino acid repeating sequence. Plasmid pV8 DNA was used to transfect wild-type vaccinia-infected CV-1 cells from which the recombinant virus V8, containing the S-antigen gene, was selected and purified.

A cDNA clone in the λgt11 amp3 expression vector (8) encoding the repeating epitope of the FC27 S-antigen has been described previously (3). The sequence of this cDNA, designated Ag16, predicts that the *P. falciparum* segment of the stable β-galactosidase-fused polypeptide must consist entirely of 11-amino acid repeats. Rabbit antibodies raised against this fused polypeptide (R210 antiserum) recognize the 200 kilodalton native S-antigen molecule (3). These antibodies reacted specifically with proteins produced by the recombinant vaccinia virus V8 in both plaque immunoassays and immunoblots of proteins isolated from V8-infected cells (Fig. 2, lanes 2 and 3). However, the apparent molecular size of the molecule was much greater than the 27 kilodaltons predicted from the sequence. Moreover, a number of less abundant smaller and larger bands were also present. The aberrant molecular weight is not due to glycosylation as proteins of the same apparent molecular weight are produced in *Escherichia coli* when the 880-bp *Aha* fragment is cloned in pUC9 under the control of β-galactosidase promotor elements to generate the plasmid pFC27Aha 2 (Fig. 2, lane 1). Moreover, the DNA insert in the recombinant virus was indeed the correct length (data not shown). We assume that abnormal SDS-binding characteristics of this protein result in this aberrant molecular weight determination on SDS-polyacrylamide gel electrophoresis. Aberrant migration is a common characteristic of plasmodial antigens containing highly repetitive sequences. Thus, the S-antigen appears to

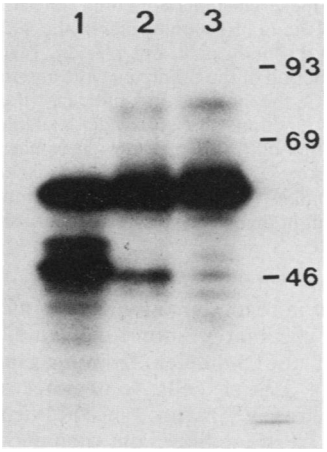

FIG. 2. S-antigen produced by recombinant vaccinia viruses is secreted. Equal fractions of V8-infected BSC-1 cells (lane 2) and the culture medium from these cells (lane 3) were collected 48 h after infection at 1 PFU per cell and subjected to immunoblot analysis. The size of the S-antigen produced was compared with that produced by *E. coli* containing the plasmid pFC27Aha2 which contains the S-antigen gene under the control of the pUC9 β-galactosidase promoter (lane 1). Samples were solubilized in SDS sample buffer and boiled before analysis by SDS-polyacrylamide gel electrophoresis and immunoblotting. Filters were probed with the polyclonal rabbit antiserum R210 which recognizes only the 11-amino acid repeat portion of the S-antigen. Molecular weights ($\times 10^3$) are shown on the right.

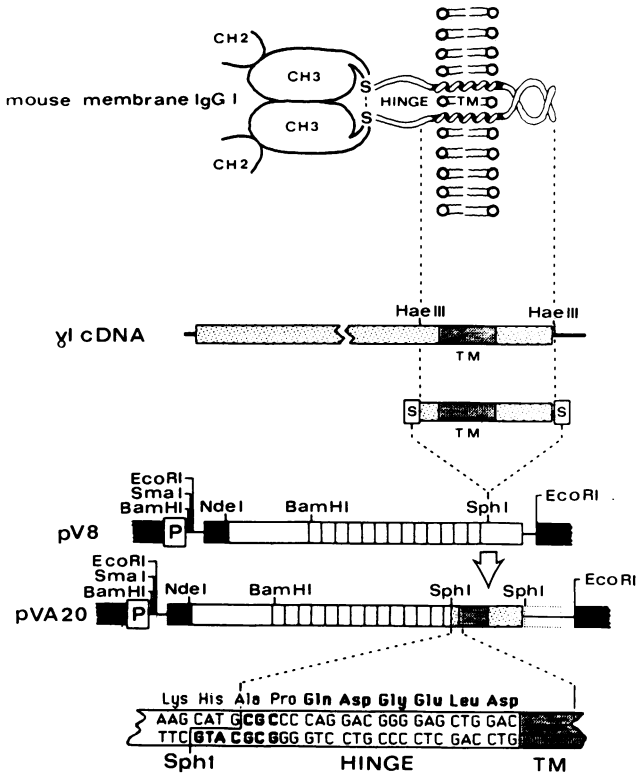

FIG. 3. Steps used in subcloning the mouse membrane IgG transmembrane sequence into the *Sph*I site at the 3′ end of the FC27 S-antigen gene. A 186-bp *Hae*III fragment encoding the transmembrane region (TM), the intracellular domain, and a portion of the hinge region was isolated from the γ1 cDNA clone described by Tyler et al. (20). *Sph*I linker DNA was added to the ends of this fragment which was then cloned into the *Sph*I site of the S-antigen gene clone pFC27Aha2 to generate the new clone pA20. The *Eco*RI fragments from these plasmids containing the S-antigen gene were then cloned into the *Eco*RI cloning site of the vector pGS62 (Fig. 1) to generate the plasmids pV8 and pVA20, respectively. The sequence at the junction of the S-antigen gene and the immunoglobulin gene is shown at the bottom and indicates the new amino acid sequence at the junction. The amino acids alanine and proline are not present in either of the parental proteins since they are generated by the *Sph*I linker DNA sequences. The six amino acids of the extracellular domain (hinge region) of the immunoglobulin gene which are present in the new hybrid protein are shown in bold-faced type. P, Promoter.

be synthesized in recombinant vaccinia-infected cells under the control of vaccinia promotor elements.

Secretion of the S-antigen from vaccinia-infected cells. Monolayers of BSC-1 cells were infected with purified recombinant virus V8. After 1 h, the virus inoculum was replaced with fresh medium, and then at various times the cells and culture medium were harvested, separated by centrifugation at 12,000 × *g* for 5 min, and subjected to analysis by immunoblotting. Detectable amounts of S-antigen began to appear in the medium at 3 to 4 h after infection and increased over the next 48 h to reach a total of over 65% of the total S-antigen synthesized (Fig. 2, lane 3). Control experiments with antivaccinia antibodies showed that the antigen present in this fraction was not due to the presence of virus in the supernatant material (data not shown). Similar results were obtained with vaccinia recombinants expressing the S-antigen of the African isolate NF7 described by Cowman et al. (4) (data not shown).

Thus, the S-antigen is secreted from the vaccinia-infected mammalian cell as it is from the parasite into the parasitophorous vacuole late in schizogony. These data indicate that the recognition signals such as the signal polypeptide are recognized despite the species differences.

Immunization of animals with V8 recombinant virus. Three rabbits were immunized as described in Materials and Methods. The antibody titers were not above preimmune levels in two of three cases (see Fig. 9, R675 and R676) and less than 1:50 in a third rabbit (see Fig. 9, R681). Antivaccinia antibodies reached high levels in all three animals. Thirteen strains of mice with three animals in each group were also vaccinated. Only marginal increases in antibody titer above preimmune values were seen at a 1:50 dilution of serum (data not shown). Rabbits immunized with recombinant vaccinia viruses expressing the antigenically distinct S-antigen of the NF7 isolate also elicited poor anti-S-antigen antibody responses (data not shown).

We concluded that despite the satisfactory expression of S-antigens in cells infected with the recombinant virus, the antigens were not recognized efficiently by the immune system of immunized animals. A possible reason for this is that the S-antigens are secreted rather than presented on the surface of the virus-infected cells.

Addition of a transmembrane sequence to S-antigen. In an attempt to retarget the S-antigen to the cell surface and hence increase its immunogenicity, a transmembrane anchor sequence was added to its carboxy terminus. A fragment of a mouse γ1 cDNA clone containing sequences encoding part of the hinge region and the whole of the transmembrane and intracellular domains of the mouse membrane IgG1 was cloned in frame into the *Sph*I site at the 3′ end of the S-antigen gene with the aid of *Sph*I linkers. The resultant hybrid gene inserted in the pGS62 vector at the *Eco*RI site is referred to as pVA20 (Fig. 3). Plasmid pVA20 DNA was used to transfect virus-infected CV-1 cells to produce the recombinant virus VA20 containing the hybrid S-antigen-IgG gene. The level of expression of the hybrid protein produced by cells infected with VA20 was similar to that of the V8 recombinant (Fig. 4, compare lanes 3 and 4 with lanes 1 and 2); however, the hybrid protein was no longer secreted from the vaccinia-infected cells (Fig. 4, compare lane 4 with lane 2).

To test whether the hybrid S-antigen containing the transmembrane segment behaved as a typical integral membrane protein, we performed Triton X-114 partition experiments (2). Whereas the V8 protein behaved exclusively as a hydrophilic soluble protein (Fig. 5, lane 1), the majority of the VA20 protein partitioned into the detergent phase (Fig. 5, lane 4), indicating that the hydrophobic transmembrane sequence had converted the soluble S-antigen protein into a membrane-associated protein.

To test whether the hybrid antigen was located on surface membranes, BSC-1 cells infected with either VA20 or V8 recombinant virus were examined 18 h after infection by indirect immunofluorescence with anti-FC27 S-antigen antibodies. Cells were either fixed before staining in cold 95% ethanol–5% glacial acetic acid to permeabilize the membranes and allow cytoplasmic labeling with antibodies or fixed after staining to reveal only surface-bound antigen. Infected cells were labeled with antiserum R210 which was obtained from a rabbit that had been immunized with the β-galactosidase fusion polypeptide Ag16 described above, followed by a fluorescein isothiocyanate-conjugated sheep antirabbit second antibody reagent. Uninfected cells did not show any labeling with the R210 antiserum (Fig. 6B, arrow).

Permeabilized cells infected with either V8 (Fig. 6A) or VA20 (Fig. 6B) showed an equally intense cytoplasmic labeling which was particularly obvious in the perinuclear region of VA20-infected cells. No labeling of the surface of unfixed, uninfected cells could be detected with the R210 antiserum. Cells infected with VA20 showed a clear pattern of surface fluorescence (Fig. 6C). V8-infected cells showed no surface labeling at low serum dilutions; however, surface labeling above background levels could be detected at higher serum concentrations (data not shown). Whether this result reflects a low level of nonspecific adsorption of the secreted S-antigen to the surface of the cells has not been investigated.

These data show that after the addition of the immunoglobulin anchor sequence to the carboxy terminus of the S-antigen gene, the hybrid S-antigen was no longer secreted. Rather, it was membrane associated and could be readily detected on the surface of recombinant virus-infected cells consistent with its identification as an integral surface membrane protein.

Immunization of animals with virus expressing the anchored form of S-antigen. The immunogenicity of the anchored S-antigen expressing the recombinant virus VA20 was compared with that of the secreted form of the S-antigen produced by V8 virus when these viruses were used to infect either mice or rabbits. In a previous experiment, a number of different strains of mice were injected intraperitoneally with 10^7 PFU of V8 virus. As mentioned above, none of the mice produced a significant level of anti-S-antigen antibody measured at either 3 weeks after a primary vaccination or 12 days after a rechallenge with an identical inoculum of virus. All mice produced good antivaccinia antibody responses, titers being in excess of 1:5,000 at 12 days after rechallenge. However, there appeared to be some differences in the antivaccinia titers between different strains measured at 3 weeks after the primary vaccination. For this reason, BALB/c.*H-2*^{k} and 129/J strains of mice (which showed moderate and high antivaccinia antibody titers, respectively) were chosen for this study. Figure 7 shows the antibody titers in serum 3 weeks after a primary inoculation with 10^7 PFU of either the V8 (open bars) or VA20 (solid bars) virus. Three weeks after primary vaccination there was an obvious increase in the amount of antibody recognizing the repeating epitope of the S-antigen in animals immunized with virus expressing the anchored form of the molecule. Interestingly, these antibody responses were higher than those found 12 days after a second immunization which was carried out 3 weeks after the primary vaccination. Antivaccinia antibody titers, on the other hand, increased dramatically in the 12 days after the challenge. There appeared to be only minor differences between the antibody responses in the two strains of mice.

Three rabbits were immunized with the VA20 recombinant virus by intradermal inoculation of 10^8 PFU of virus and rechallenged 6 weeks later. A typical response is described in Fig. 8. Antivaccinia antibody titers increased over the period of analysis and were boosted by a challenge with virus 6 weeks after the primary immunization. Anti-S-antigen antibody titers increased to a maximum in the second week after immunization and then waned rapidly. Rechallenging the rabbits at 6 weeks after the primary immunization resulted in a marked increase in the antivaccinia antibody level but had a negligible effect on the level of anti-S-antigen antibody. These data are in accord with those obtained in mice.

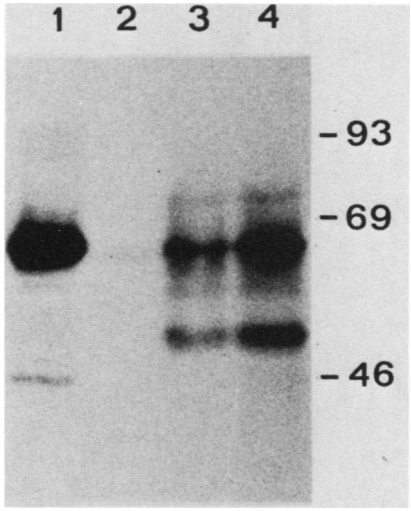

FIG. 5. S-antigen produced by cells infected with the VA20 recombinant virus partitions like an integral membrane protein in Triton X-114. BSC-1 cells infected for 48 h with either the V8 (lanes 1 and 2) or VA20 (lanes 3 and 4) recombinant virus were solubilized in 0.05% Triton X-114 for 1 h at 4°C. Insoluble material and nuclei were removed by low-speed contrifugation. After incubation for 5 min at 37°C, a cloudy suspension of insoluble Triton X-114 micelles was separated by centrifugation at 37°C, and each fraction was repurified by a further cycle of Triton X-114 partitioning. The S-antigen present in the aqueous (lanes 1 and 3) and detergent (lanes 2 and 4) phases was detected by immunoblotting with the rabbit antirepeat antiserum (R210). Molecular weights ($\times 10^3$) are shown on the right.

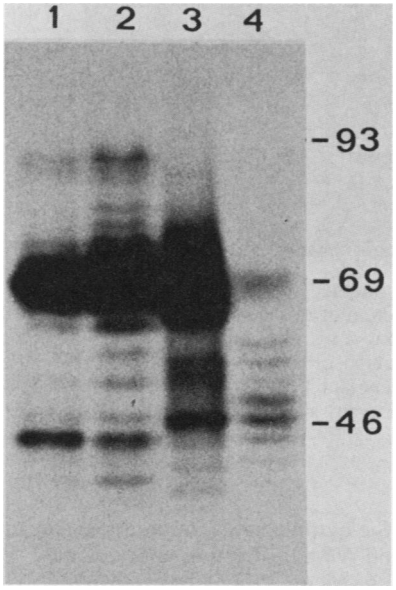

FIG. 4. S-antigen produced by cells infected with the VA20 recombinant virus is no longer secreted. Samples of infected cells (lanes 1 and 3) and culture medium (lanes 2 and 4) were collected 48 h after infection with either the recombinant virus V8 (lanes 1 and 2) or VA20 (lanes 3 and 4). The total amount of S-antigen detected in lanes 1 and 2 appears to be similar to that detected in lane 3. However, very little is secreted into the medium in the case of VA20-infected cells (lane 4). Immunoblots were probed with the rabbit antirepeat antiserum R210. Molecular weights ($\times 10^3$) are shown on the right.

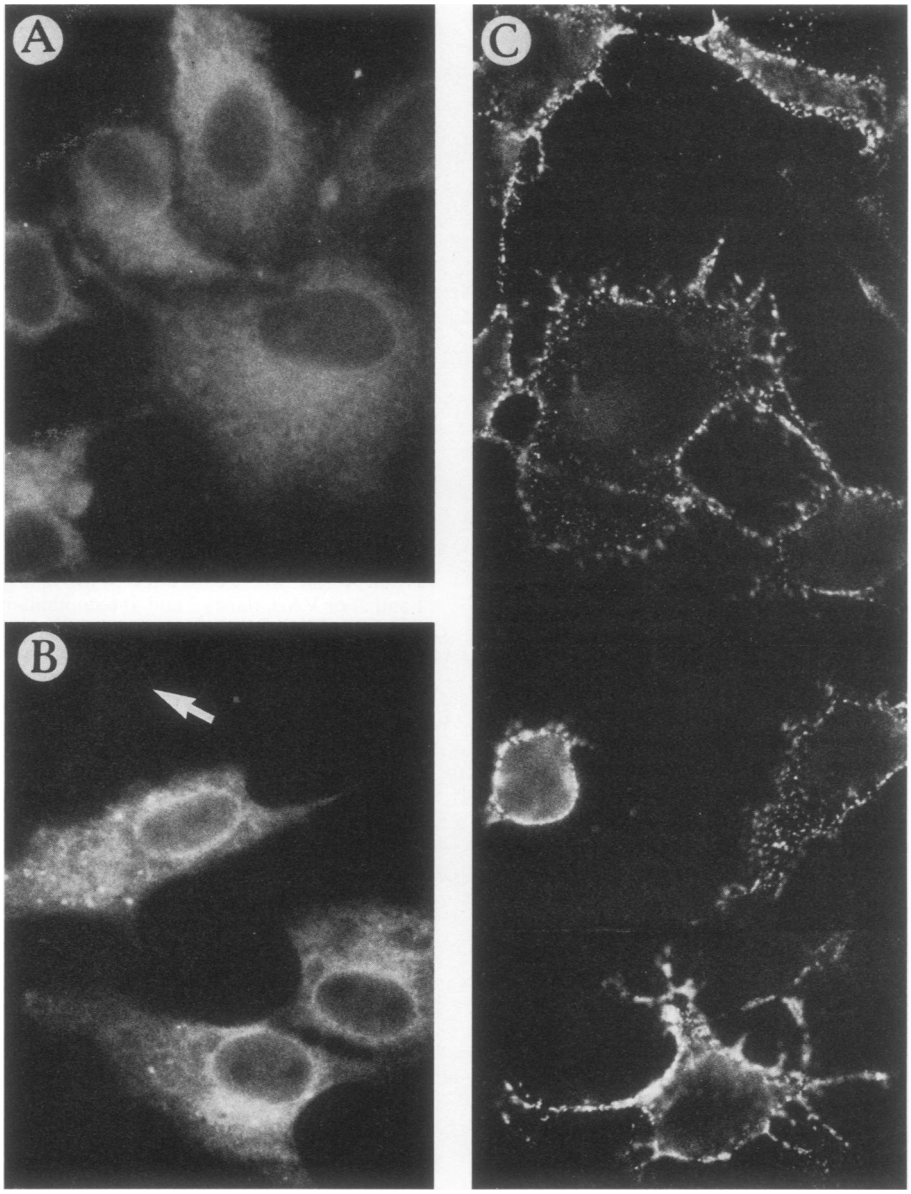

FIG. 6. Localization of S-antigen by indirect immunofluorescence of BSC-1 cells infected with V8 (A) or VA20 (B and C) recombinant virus. Cells were either fixed before staining to permeablize the cells and allow detection of intracellular S-antigen (A and B) or fixed after staining to detect S-antigen localized on the surface of the infected cells (C). Fixation was with ice-cold 95% ethanol–5% glacial acetic acid. Rabbit R210 antiserum at a 1:500 dilution was used to localize the S-antigen. A fluorescein isothiocyanate-conjugated goat antirabbit antibody was then used as the second label before mounting in glycerol containing the fluorescent stabilizer 1,4-diazobicyclo-2,2,2-octane. Cells were photographed under UV illumination and oil immersion. Uninfected cells did not label to any significant extent as shown by the cell indicated with an arrow in panel B.

The sera of rabbits showing the highest anti-S-antigen titers were assayed in parallel with the highest titered sera from rabbits immunized with V8 which also showed a peak response at 2 weeks after the primary challenge (Fig. 9). The anti-S-antigen antibody response was clearly enhanced in rabbits immunized with the VA20 recombinant when compared with the response in V8-infected animals.

Deleting the signal peptide sequence from the S-antigen gene results in rapid degradation of the S-antigen. An attempt was made to assess the relative immunogenicity of a cytoplasmically located S-antigen protein in comparison with the secreted and membrane-anchored forms. The S-antigen was targeted to the cytoplasm by deleting the sequences between the *Sma*I and *Nde*I restriction endonuclease sites shown in Fig. 1 which encode the 5′ untranslated region and the majority of the signal peptide. These sequences were replaced with the short oligonucleotide sequence 5′-CACCATGGTG-3′ which generates a new in-frame initiation signal flanked by sequences typically found at the site of initiation of translation of eucaryotic genes (9). This resulted in the deletion of 21 of the 23 amino acids of the signal peptide sequence of the S-antigen molecule which were replaced by only 2 new amino acids (including the initiation methionine). This protein should be only three amino acids

longer than that remaining after cleavage of the signal peptide from the parental molecule.

Recombinant vaccinia viruses expressing this S-antigen lacking the signal peptide were selected in a plaque immunoassay with rabbit R210 antiserum. Western blot analysis (data not shown) indicated that the S-antigen molecule had been degraded into small peptides which migrated with the dye front on a 10% polyacrylamide–SDS gel. Rabbits and mice immunized with this recombinant virus produced low antibody titers to the S-antigen similar to those produced with the secreted S-antigen construct (data not shown). Because the S-antigen is degraded and is therefore no longer analogous to the molecules being presented by the virus-infected cells expressing the secreted and membrane-anchored forms of the antigen, no comparison can be made between the immunogenicity of a cytoplasmic and a cell surface-localized antigen in this experiment. It does indicate, however, that the presence of the signal peptide and therefore the transport of this protein into the lumen of the endoplasmic reticulum confers a degree of stability to this foreign antigen in virus-infected cells.

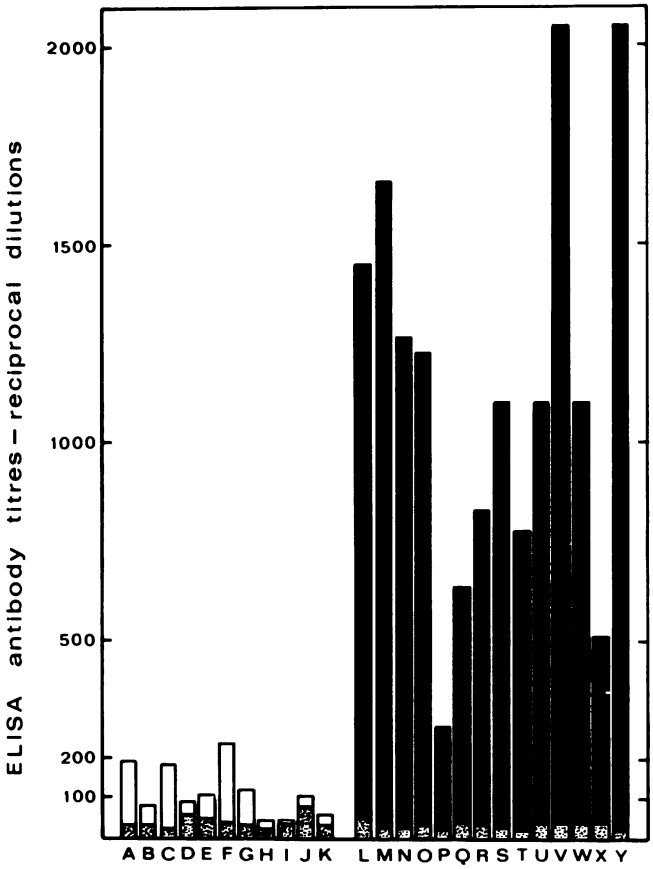

FIG. 7. Anti-S-antigen antibody titers in immunized mice determined by ELISA. Mouse sera were assayed at 3 weeks after a single intraperitoneal immunization with 10^7 PFU of the V8 (mice A to K) or VA20 (mice L to Y) recombinant virus. Microtiter trays (96 wells) were coated with an FC27 S-antigen repeat-β-galactosidase fusion polypeptide preparation (Ag16) at a predetermined optimal concentration of 5 μg/ml. The titers for preimmune (speckled shading) and immune (open and solid shading) sera were determined from the serum dilution at which half maximal absorbance in the ELISA was reached. These values represent titers plotted for both the BALB/c.*H-2*^{k} (mice A to G and L to R) and 129/J (mice H to K and S to Y) strains of age-matched female mice.

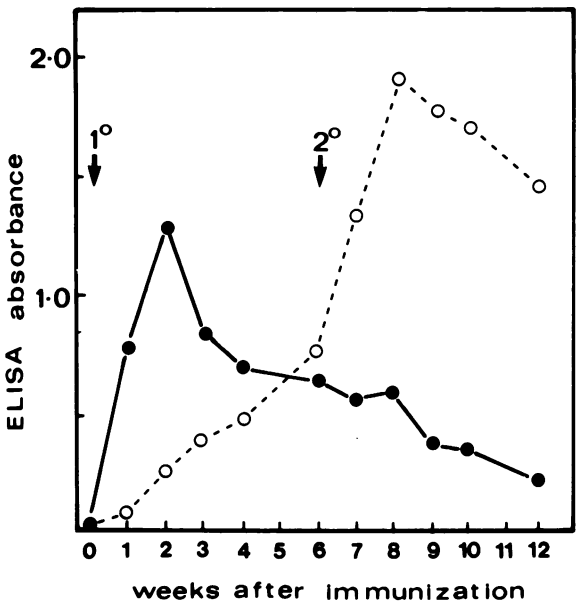

FIG. 8. Time course of anti-S-antigen and antivaccinia antibody titers in a rabbit immunized with VA20. A plot of the absorbance values obtained in a typical ELISA of rabbit antiserum R754 taken at weekly intervals after a single intradermal injection of 10^8 PFU of live recombinant virus VA20 (1°) which was followed 6 weeks later by an identical challenge dose (2°). Sera were assayed both for anti-S-antigen antibodies as described in the legend to Fig. 7 at a standard dilution of 1:320 of the serum (●) and for antivaccinia antibodies at a standard dilution of 1:6,400 of the serum (○).

DISCUSSION

The data presented here on the expression of the S-antigen show that the signals which direct secretion are recognized accurately when a secreted plasmodial protein is expressed in vaccinia-infected cells. Immune responses to the secreted form of the S-antigen in animals immunized with the recombinant virus were poor. Most foreign antigens which have been expressed in recombinant vaccinia viruses have elicited good immune responses, although in the majority of cases the antigen was expressed on the surface of the virus-infected cells. Interestingly, the hepatitis B surface antigen which is not inserted into the surface of vaccinia virus-infected cells but is secreted in the form of 22-nm particles was a good immunogen in rabbits but produced no antibody responses in chimpanzees even though these animals were protected against hepatitis B infection (13, 18).

A number of other plasmodial antigens have been expressed in recombinant vaccinia viruses none of which have been transported to the surface of the virus-infected cell. The circumsporozoite coat protein genes from *P. knowlesi* (17) and *P. falciparum* (unpublished observations) produce proteins which are larger than the native molecule and, although not located on the surface of virus-infected cells, induce a measurable antibody response in rabbits. These results also indicate that at least some of the signals that direct plasmodial surface antigens to their target membranes may not be correctly interpreted by the processing machinery of the mammalian cell. We have also obtained similar results with the ring infected erythrocyte antigen-vaccinia recombinants (L. M. Corcoran, unpublished observations). It appears that an immune response can be generated to an antigen which is not expressed on the surface; however, the

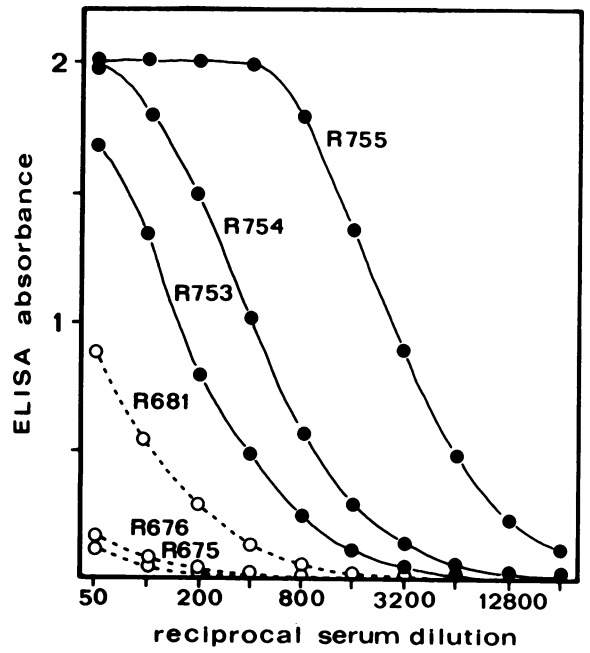

FIG. 9. Anti-S-antigen antibody responses in immunized rabbits. A plot of the anti-S-antigen antibody levels obtained in a typical ELISA of rabbit antisera taken at 2 weeks after a single intradermal injection of 10^8 PFU of VA20 recombinant virus (rabbits R753, R754, R755 [●]) or V8 recombinant virus (rabbits R675, R676, R681 [○]). Serial dilutions of the sera were assayed in duplicate in plates coated with the Ag16 fused polypeptide at a concentration of 5 μg/ml.

data presented here suggest that this response would be small in comparison to what might be produced if they were presented as surface antigens.

This report describes the successful targeting of a secreted antigen to the cell surface by the addition of the carboxy-terminal region of a mammalian transmembrane protein, the mouse immunoglobulin γ1 chain. This approach may facilitate the more optimal presentation of other malarial and nonmalarial antigens which would otherwise not be expressed on the surface of live recombinant virus-infected cells. However, attempts by others to express hybrid molecules on the cell surface indicate the need for some caution before assuming that this procedure has general applicability.

Initial attempts to redirect the secreted rat growth hormone protein to the cell surface by the addition of the transmembrane and cytoplasmic domains of the vesicular stomatitis virus glycoprotein G failed (7). In this case the hybrid protein, expressed from transfected DNA in COS cells, became associated with the membranes of the reticular system and Golgi apparatus as an integral membrane protein but was not transported to the cell surface. Clearly, it cannot be assumed that a secreted protein will necessarily be redirected to the cell surface by the simple addition of a hydrophobic membrane-spanning region and intracellular domain from another protein.

In the case of the hybrid rat growth hormone-vesicular stomatitis virus G protein, it was subsequently shown that the block in the transport to the cell surface could be overcome by the addition of N-linked glycosylation sites into the amino acid sequence of the rat growth hormone protein (6). The authors use this observation to conclude that the presence of N-linked glycans may be a critical signal for the transport of proteins to the cell surface. In this respect it is interesting to compare the structure of the hybrid proteins described by Guan and Rose (7) and in this report. Both the native S-antigen and rat growth hormone lack potential N-linked glycosylation consensus sequences, whereas the vesicular stomatitis virus G protein and the mouse immunoglobulin protein both contain extensive complex carbohydrates. That one hybrid should be successfully transported to the cell surface and the other not demonstrates that we do not fully understand the subtle differences that define the altered pathways a protein may take in its passage from its place of synthesis to its final cellular location. It is possible that the inclusion of potential N-linked glycosylation sites into the hybrid S-antigen protein may further improve its cell surface expression and hence its immunogenicity.

Other refinements of the recombinant vaccinia virus presentation system may result in the further enhancement of the immune response to the foreign antigen. The use of stronger vaccinia promotors resulting in the higher expression of the introduced protein is one obvious method. Insertion of the foreign gene at a different location in the vaccinia genome may give rise to a less attenuated virus and therefore an increased immune response. Recombinant viruses expressing adjuvant molecules as well as the foreign antigen may further stimulate the immune response. As well, this may decrease the spread of the virus, thus reducing the risk of side effects which sometimes result from poorly controlled viremias.

The addition of the anchor sequence resulted in a marked increase in the levels of antibodies recognizing the repeating epitope of this antigen. It is unlikely that the addition of the mouse immunoglobulin anchor sequence caused this effect simply by altering the tertiary structure of the protein since evidence, including data from the use of monoclonal antibodies, suggests that the repeats of the native S-antigen molecule are recognized as linear determinants. However, the Ag16 fused polypeptide which contains 23 copies of the FC27 S-antigen repeat at the carboxy-terminal end of β-galactosidase induced good antibody responses in rabbits and mice after a series of immunizations in Freund adjuvant. One possibility is that the β-galactosidase provides carrier epitopes which, by activating T helper cells, increase the antibody response to the S-antigen repeats. It is unlikely that the mouse IgG anchor sequence performs this same function because these sequences are "self" in the mouse immunization studies and the magnitude of the increased antibody responses observed in rabbits was the same as that for mice. This is strong evidence that the surface location is the cause of enhanced immunogenicity.

The use of an S-antigen in these experiments is of added interest because of unresolved questions concerning the immunogenicity of these unusual repetitive proteins. The particularly poor immune response to the secreted S-antigen may be related to its secretion and clearance as well as to the inherent immunological properties of this molecule. Antibodies to S-antigens are much more prevalent in adults than in children in malaria endemic areas, but the incidence of patients with antibodies to a particular S-antigen serotype is lower than one would predict from the incidence of parasites expressing that S-antigen type in a defined geographical region. However, this may be due to the transient nature of anti-S-antigen antibody responses in humans, although there are as yet no data to support this notion. Rarely, adult immune sera contain very high anti-S-antigen titers, and usually these sera contain antibodies to more than one

serologically distinct S-antigen type. Furthermore, it has so far not been possible to raise antibodies to partially purified S-antigens isolated from in vitro malaria cultures by immunizing laboratory animals (21; unpublished observations).

It is possible, therefore, that the S-antigens because of the highly conserved sequence of the repetitive structure and their soluble nature are not under normal circumstances very immunogenic in vivo and that only after repeated malarial attacks do readily detected antibodies to this group of molecules develop. The data presented here are consistent with both these possibilities. Antibody responses to the secreted S-antigen expressed by recombinant vaccinia virus in infected rabbits and mice are poor. These responses are not boosted by challenging with a second immunization with recombinant virus. Antibody titers peak early after infection and wane rapidly, even after the addition of the membrane anchor sequence, suggesting that the low antibody responses to these S-antigen proteins are not solely related to their secreted nature.

As yet no function has been ascribed to S-antigens. They do not appear to be located or attached to the surface of merozoites or erythrocytes at any stage although it cannot be excluded that there is a very weak attachment which is too labile to visualize histologically or to demonstrate experimentally. However, their extreme serological diversity suggests that their functions are in some way related to immune evasion. If S-antigen function is to modulate the immune responses to the advantage of the parasite then such modulations are certainly not achieved by a process of overstimulation of B-cell activity as has been suggested for some malarial antigens (1). Why S-antigens, the most variable of all the known antigens of *P. falciparum*, should be poorly immunogenic and therefore under reduced immune pressure to vary, remains a conundrum. However, the use of vaccinia virus as an immunizing vector may permit a dissection of their remarkable immunological properties.

In summary, our data indicate the importance of presentation of foreign antigens on the surface of live recombinant vaccinia virus-infected cells for the increased production of antibodies to the foreign antigen. This report also described an approach by which any malarial antigen which is not normally expressed on the cell surface could be displayed on the surface of vaccinia-infected cells as a hybrid molecule. Alternatively, oligonucleotides encoding critical (linear) epitopes from a number of antigens could be ligated together and, in association with the appropriate signal sequences, be expressed on the surface of virus-infected cells. This strategy may be useful in the production of a multivalent subunit vaccine which would incorporate the advantages of a live virus presentation system.

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