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DEVELOPMENT OF A SPOROZOITE MALARIA VACCINE

VICTOR NUSSENZWEIG & RUTH S. NUSSENZWEIG

In this lecture, which I also delivered at the recent meeting of the American Society of Tropical Medicine and Hygiene (*Am. J. Trop. Med. Hyg.*, *35* :678-688, 1986). I will be the spokesman, not only for Ruth, but also for a large number of colleagues and students at several institutions who collaborated in the sporozoite vaccine project. I will summarize their work and discuss some of the unusual characteristics of circumsporozoite proteins and their genes. I will also present recent results of Drs. Arturo Ferreira and Louis Schofield showing that the development of exoerythrocytic forms (EEF) of rodent and human malaria parasites can be strongly inhibited *in vivo* and *in vitro* by lymphokines.

In relation to the development of the malaria vaccine, the key finding by Ruth, J. Vanderberg and their co-workers was that the repeated injection of small numbers of x-irradiated, attenuated sporozoites of *Plasmodium berghei* into mice protected them against challenge with viable sporozoites. Similar observations were made shortly afterwards by Drs. Allan Cochrane and R.W. Gwadz in monkeys immunized with irradiated *P. knowlesi*. Then Dr. Clyde and his collaborators performed their already classical experiments in which a few human volunteers were succesfully vaccinated with sporozoites of *P. falciparum* or *P. vivax*. This vaccine, by the way, was not a recombinant or synthetic vaccine. It was delivered by the bite of infected irradiated mosquitoes!

The next important observation was made again by Ruth and collaborators, who showed that the serum of the protected animals neutralized sporozoite infectivity, which implied that protection was at least in part antibody-mediated. When Jerry Vanderberg mixed the serum of the protected mice with sporozoites he observed what is called now the circumsporozoite (CSP) reaction (Fig. 1), that is, the appearance of heavy deposits on the parasite surface, followed by the formation of a long tail, which represents the shedding of the immune complexes. Taken together, these two observations suggested that the antigen involved in the CSP reaction might be involved in protective immunity.

The next logical step was to characterize the sporozoite surface antigens by means of monoclonal antibodies. This was the work of Drs. Yoshida and Potocnjak, two postdoctoral students. The first monoclonal antibody raised against the surface membrane of *Plasmodium berghei* mediated the CSP reaction. It reacted with a polypeptide of M_r 44,000 (Pb44) which covered the whole surface of sporozoites, as shown by immunoelectronmicroscopy studies of Dr. Aikawa. More important, the intact monoclonal antibody, or the Fab fragments, neutralized sporozoite infectivity. At the time when these studies were performed the infectivity of sporozoites had to be measured by microscopic examination of blood smears taken daily after the intravenous injection of the parasites. Because the dose response relating the number of injected sporozoites *versus* prepatent periods is extremely flat, this is an insensitive and non-quantitative way of evaluating sporozoite infectivity. Recently a specific DNA probe for *P. berghei* isolated by Dr. V. Enea at NYU was used by Dr. Arturo Ferreira to measure EEF in livers of rats injected with sporozoites. Using this method he could also determine with precision the neutralization capacity of the monoclonal antibodies against CS proteins. Various doses of the antibody were incubated with 10^5 *P. berghei* sporozoites, and injected into rats. The livers of the animals were removed when the development of the EEF was maximum, that is, 44 hours after injection. Liver DNA was isolated and the amount of parasite DNA measured with the specific probe. Dr. Ferreira found that less than 10 micrograms of antibody neutralized more than 98% of injected parasites.

Several additional observations in the monkey and human malaria models suggested that CS proteins are involved in the initial parasite interaction with the target hepatocytes. In the first place CS proteins are the major (or only) protein found on the surface of sporozoites. More important, Fab fragments of antibodies to the CS proteins inhibit the infectivity of sporozoites *in vivo* and *in vitro*. Antibody-treated sporozoites do not adhere to hepatocytes *in vitro*. Also compatible with the hypothesis that CS proteins are functionally important to the survival of

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Departments of Pathology and Medical and Molecular Parasitology – New York University Medical Center, New York, NY 10016, USA.

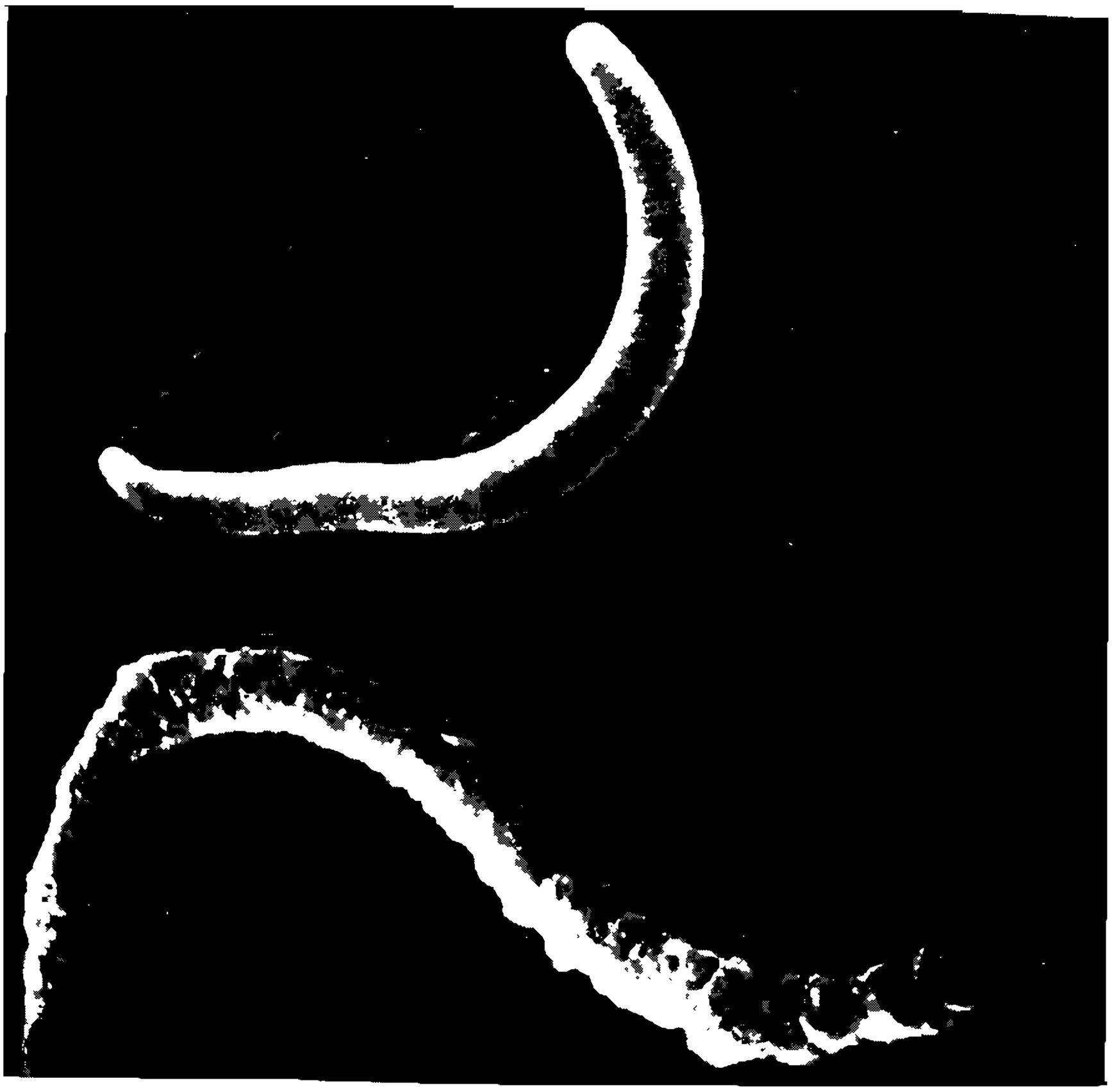


Fig. 1: Scanning electronmicrograph of sporozoites incubated with immune serum (botom), or with normal serum (top) (Dr. M. Aikawa).

sporozoites in the mammalian host is the fact that CS proteins are stage-specific and are synthesized in larger amounts only in mature salivary gland sporozoites. Mid-gut parasites have less or no CS protein and are not infective.

Subsequent studies comparing the structural, immunological and biological properties of CS proteins showed that they were part of a distinct family of proteins. CS proteins cover uniformly the surface of the sporozoites and are shed when cross-linked by antibodies. The studies of Drs. Yoshida, Cochrane and Nardin showed that all CS proteins are synthesized in large amounts by mature sporozoites. The M_r varies between 40 and 60,000 and isoelectric points under denaturing conditions between 4 and 5. They have two intracellular precursors of higher M_r, but the processing steps leading to the insertion of the CS protein into the membrane of sporozoites remain to be clarified. Some of the most remarkable properties of CS proteins were uncovered by Dr. Zavala. He found that all monoclonal and polyclonal antibodies to these relatively large polypeptides were directed against a single epitope. Moreover, this unique epitope was represented more than once per molecule, since each could simultaneously bind at least two molecules of the monoclonal antibodies.

Because the amounts of pure CS protein necessary for vaccination could not be obtained from sporozoites, but had to be obtained by genetic engineering methodology, we decided that it was essential to identify the CS gene. *P. knowlesi* sporozoites were chosen as the target of the cloning effort because they are abundant in the salivary glands of *Anopheles dirus* mosquitoes. About 10^8 sporozoites from infected mosquitoes raised by Dr. R. Gwadz, were given to a graduate student, Joan Ellis. In cooperation with Dr. Nigel Godson, Joan obtained mRNA, prepared a cDNA library, and used a sensitive two-site immunoradiometric assay developed by Dr. Zavala to identify a clone expressing a part of the CS gene. Through the work of Drs. Ozaki and Godson, a genomic copy of the CS gene was obtained.

The structure of the CS protein as deduced from the nucleotide sequence of the structural gene is schematically shown in Fig. 2. The essential features are conserved in the CS proteins from

other monkey or human malaria parasites, identified later by Drs. Enea and Arnot at NYU, and Dame, McCutchen and collaborators at the NIH. All contain hydrophobic N-terminal and C-terminal sequences, which presumably represent signal and anchor sequences; two areas with a large proportion of charged amino acids, one of them closer to the C-terminal and containing two pairs of cysteine residues; and a large domain consisting of tandemly repeated sequences of identical amino acids. (As shown in Fig. 2, in *P. knowlesi* this domain contains 12 amino acids repeated 12 times.) All monoclonal antibodies which neutralize sporozoite infectivity are directed against the repeats of the various CS proteins, including those of *P. falciparum* and *P. vivax.*

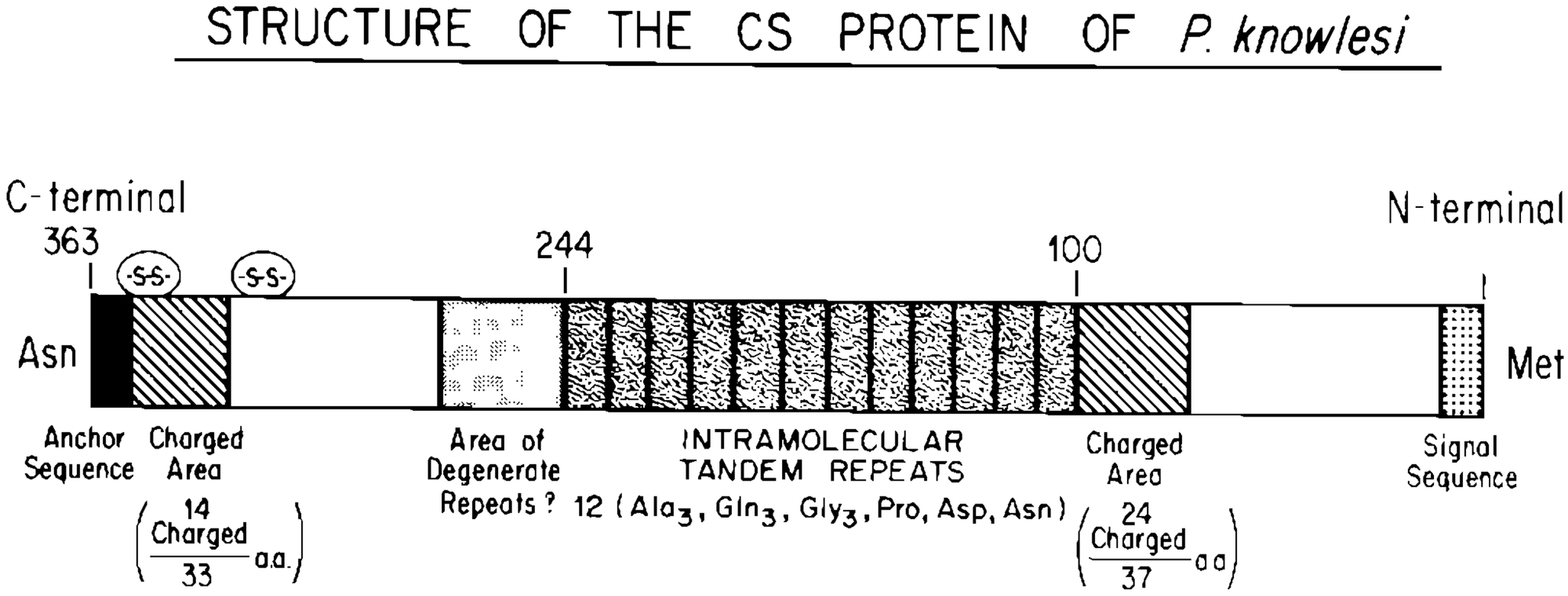


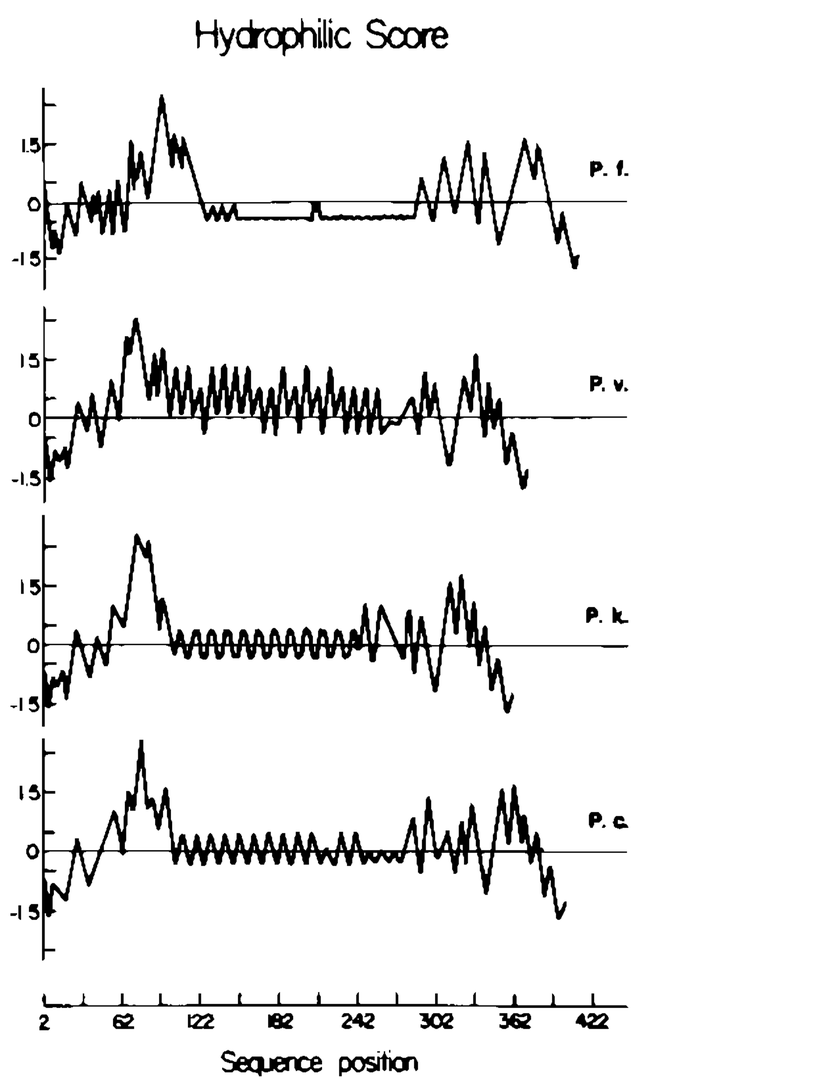
Fig. 2: Schematic representation of the CS protein of *P. knowlesi* as deduced from the nucleotide sequence of the corresponding gene. Approximately 40% of its 363 amino acids consist of tandem repeats of 12 amino acids. At the N-terminal and C-terminal there are stretches of hydrophobic amino acids that probably constitute signal and anchor sequences. Areas containing large numbers of charged amino acids are found on either side of the repeats. One of these (24 charged amino acids among 37) flanks the repeats in the direction of the N-terminal. The other charged area (14 charged amino acids among 33) is closer to the C-terminal and is situated between two pairs of cysteine residues, each pair previously implicated in the formation of an intramolecular loop. The numbers represent the position of amino acids in the sequence, starting at the N-terminal.

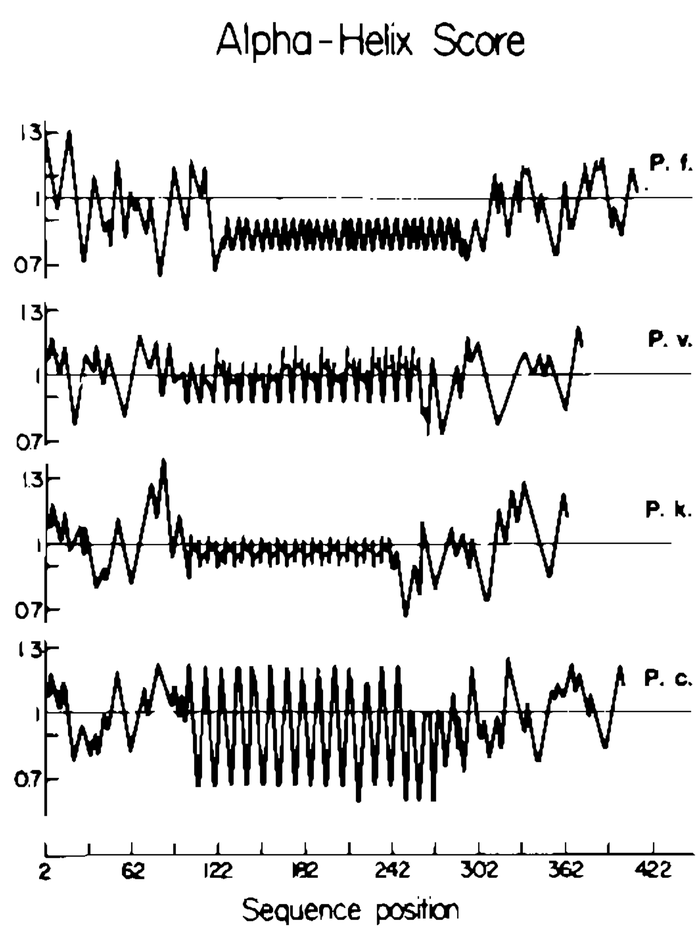
To obtain additional information about the structure of the CS proteins, the amino acid sequences were fed into computers and analyzed by programs designed to estimate their hydrophilicity and the probability that they will form secondary structures, α-helices, β-sheets, or reverse turns in the native protein.

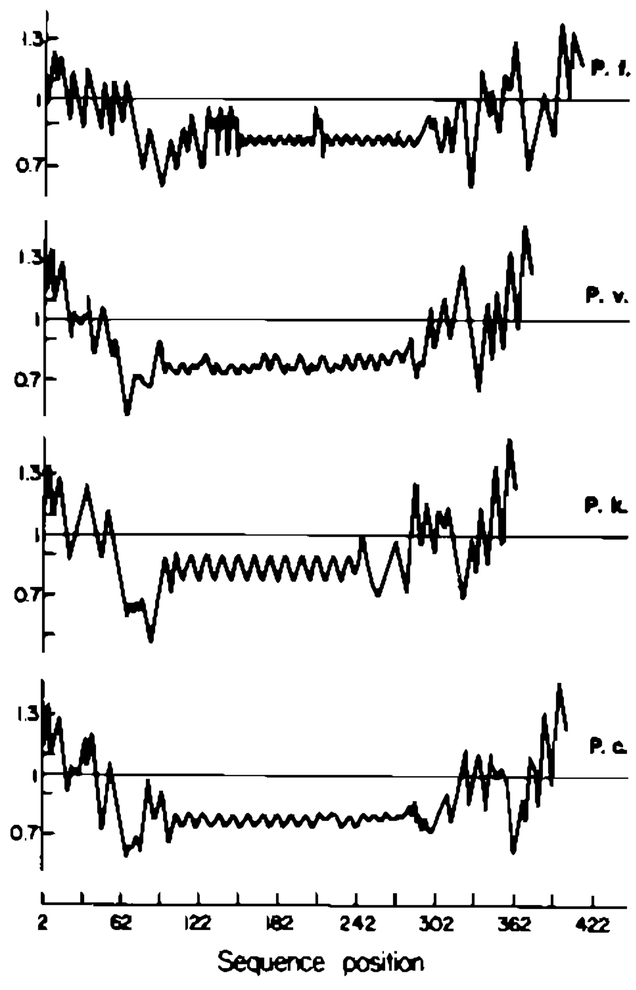
The results were quite informative. In Fig. 3 (top, left) the amino acid sequences of the CS proteins of *Plasmodium falciparum, vivax, knowlesi* and *cynomolgi* are analyzed for hydrophilicity. The two areas containing charged amino acids on both sides of the repeats have positive scores. The repeats are in the gray area, neither very hydrophilic, nor hydrophobic. On the basis of commonly used criteria to forecast the immunogenicity of proteins, many would have incorrectly predicted that the charged areas would be more immunogenic than the repeats! Sporozoites are teaching immunologists a lesson, that is, that tandem repeated sequences can enhance antibody production, provided other essential requirements are met, such as, for example, surface accessibility.

Other predictions about the secondary structure of CS proteins are also shown in Fig. 3. The repeats do not show any tendency to form α helices (top, right) or β sheets (bottom, left) but the β turn (or reverse turn) score (bottom, right) is, in every case, high. This may be important for the development of synthetic vaccines because reverse turns are simple secondary structures which depend exclusively on primary amino acid sequences. A synthetic peptide containing the sequence of a reverse turn is more likely to have a reverse turn configuration in solution and to mimic more faithfully the structure of the same sequence in the native protein. It should be also pointed out that in the case of *P. falciparum,* the repeat subunit is exceedingly simple (asparagine – alanine – asparagine – proline) and that two amino acids (asparagine and proline) are frequently found in reverse turns in native proteins.

These observations convinced us that it was reasonable to try to develop a synthetic vaccine against *P. falciparum* sporozoites containing NANP repeats. Now, the first question that







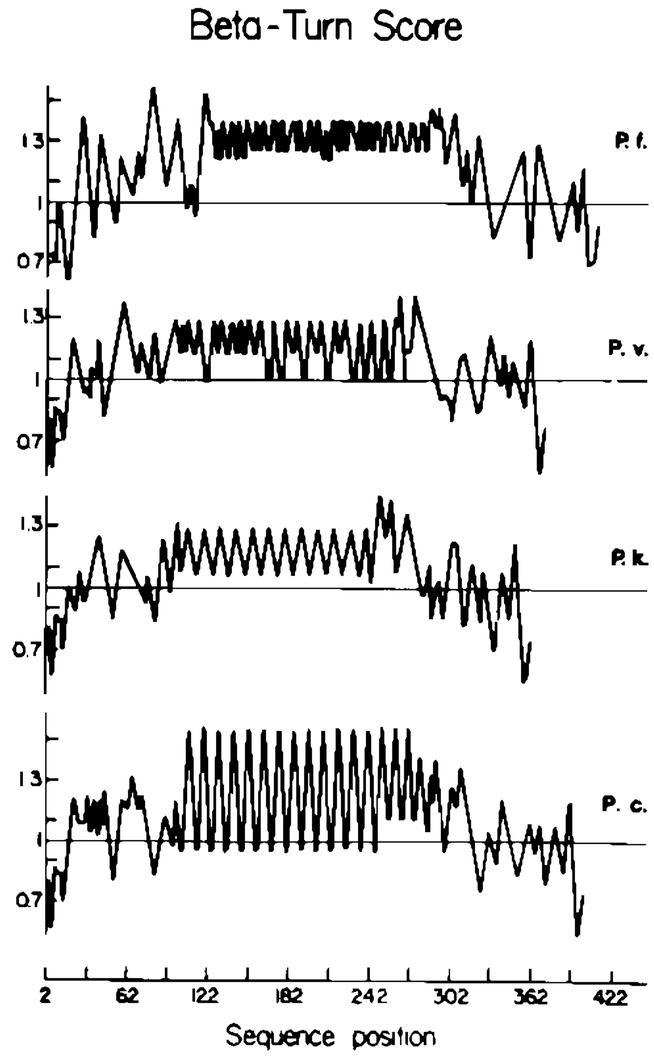


Fig. 3: Analysis of the amino acid CS protein sequences of ***P. falciparum*** (P.f.), ***P. vivax*** (P.v.), *P. knowlesi* (P.k) and *P. cynomolgi* (P.c.) by the Chou-Fasman method of predicting secondary structures, as adapted by the Protein Identification Resource, National Biomedical Research Foundation, Washington, DC 20007.

we faced was, "how many NANP tandem repeats form an epitope which can be recognized by most antibodies to sporozoites?" To answer this question we had the collaboration of Dr. James Tam (Rockefeller University), who synthesized a series of small peptides containing tandem repeats, $(NANP)_1$, $(NANP)_2$, $(NANP)_3$, $(NANP)_4$, and $(NANP)_5$. Dr. Zavala then compared their ability to inhibit the reaction of various monoclonal antibodies with the native CS protein. The results were clear-cut and showed that $(NANP)_2$ was a poor inhibitor while $(NANP)_3$, $(NANP)_4$, or $(NANP)_5$ were equally effective (Fig. 4). We concluded that the epitope recognized by the monoclonal antibodies was contained within $(NANP)_3$.

To confirm that $(NANP)_3$ also represented the main epitope recognized by human antibodies, Dr. Zavala analyzed the patterns of reactivity of sera from individuals living in endemic areas. Samples were collected randomly from children and adults in the Gambia, and an immunoradiometric assay (IRMA) was performed using $(NANP)_3$ as antigen. Indirect immunofluorescence (IFA) assays to detect antisporozoite antibodies were then performed in randomly selected IRMA positive sera from adults. The results (Table I) showed a very good correlation between the IRMA and IFA titers ($r_S = 0.87$, $P < 0.001$). Moreover, the $(NANP)_3$ peptide in solution inhibited almost

100% of the reactivity of the sera with the parasite surface. This finding is remarkable because it implies that the immune response of individuals who are repeatedly exposed to the bite of mosquitoes infected with sporozoites is directed mainly or exclusively to $(NANP)_3$.

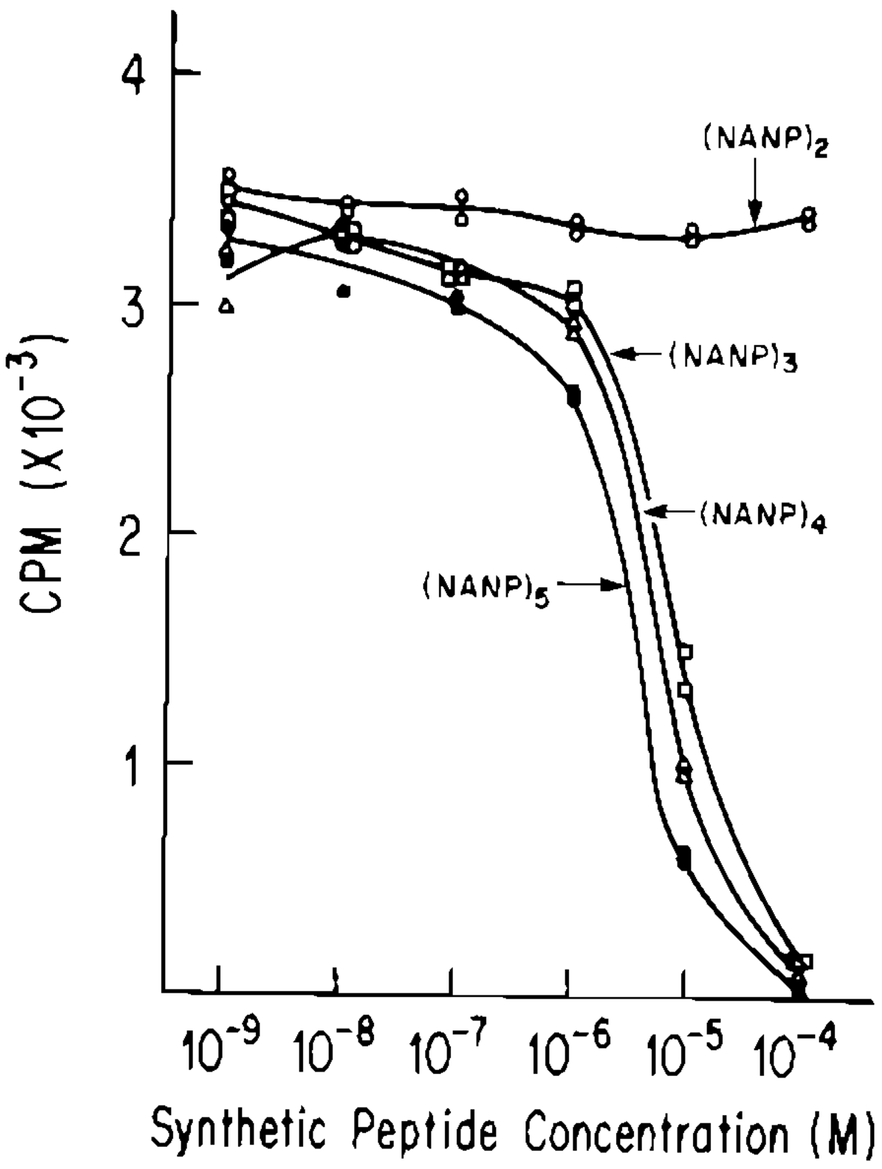


Fig. 4: Inhibition of binding of a monoclonal antibody against *P. falciparum* sporozoites by synthetic peptides. $(NANP)_2$, $(NANP)_3$, $(NANP)_4$, $(NANP)_5$. The antibody 2A10, at a concentration of 50 ng/ml was incubated with increasing concentrations of peptide. After 1 hour at room temperature, 30 μl of the mixtures were placed in duplicate wells of *P. falciparum* sporozoite-coated plates. After incubation for 1 hour, the wells were extensively washed with phosphate-buffered saline (PBS) containing 1 percent of bovine serum albumin (BSA) and 0.05 percent Tween-20. Then 30 μl of ^{125}I-labeled affinity purifed goat antibody to mouse immunoglobulin were placed in each well, incubated for 1 hour, washed three times with PBS-BSA, dried, and counted in a gamma counter.

$(NANP)_3$ was then coupled to tetanus toxoid, a carrier protein which can be injected into humans, and the conjugate used to immunize rabbits. All rabbits made antibodies to sporozoites in high titers and the antibodies neutralized sporozoite infectivity *in vitro*. The result of one such experiment performed by Dr. Hollingdale is shown in Table II. The IgG fraction from the immune serum prevented the entry of the parasite into the target cell in a dose-dependent fashion. A strong effect was observed at a concentration of IgG of 2μg/ml. When the antibodies to $(NANP)_3$ were removed by immunoabsorption, the activity was abolished.

As you probably know, two vaccines containing the *P. falciparum* NANP repeats have been manufactured. One of them is undergoing phase one human trials now and the other will be injected into humans soon. It will be of great interest to compare the efficiency of these two new types of vaccines, one made by genetic engineering (Smith, Kline & French) and the other containing synthetic peptides (Hoffmann-La Roche). One of the most attractive features of this vaccine is the apparent lack of variation in the *P. falciparum* repetitive epitope. All strains which were examined by Zavala and by Weber and Hockmeyer contain this epitope. Therefore, if effective, a single preparation could be used all over the world. On the basis of our experimental results, Ruth and I hope that these vaccines will confer some degree of protection against *P. falciparum* malaria.

TABLE I

Specificity of antibodies to *P. falciparum* sporozoites in the sera of randomly selected individuals older than 20 years of age and living in a malaria endemic area

Serum	IRMA with $(NANP)_3$ as antigen (Δcpm)*	IFA with glustaraldehyde-fixed sporozoites as antigen	
		Serum titer	Serum titer with $(NANP)_3$ ω
G.Z.♂	9201	4096	320
IDA	4851	1280	<20
8017	3539	640	<20
7930	3501	640	<20
7979	3311	640	<20
7973	2735	320	<20
P-2	2473	320	<20
P-5	2024	320	<20
8012	1765	640	<20
Normal	163	<10	ND
7981	168	<10	ND
8074	133	20	ND
7878	96	<10	ND
P-12	75	<10	ND
8312	72	<10	ND
P-11	−13	<10	ND
8286	91	20	ND
7907	−103	<10	ND

* The IRMA antigen was $(NANP)_3$ immobilized by means of glutaraldehyde in plastic wells coated with BSA. To saturate the remaining glutaraldehyde reactive groups, the wells were treated with 0.5M ethanolamine. Serum samples were ten times diluted in a mixture of BSA and PBS containing 0.5M ethanolamine and 0.5 percent Tween-20, and 20μl were placed in each well. After 1 hour, the wells were washed and incubated with ^{125}I-labeled affinity-purified goat antibodies to human immunoglobulins to reveal the presence of bound antibodies. Each serum was simultaneously tested in duplicate peptide-coated wells and in control wells prepared as described above but omitting the peptide. The mean radioactivity in control wells, which varied from 200 to 500 count/min, was subtracted from the radioactivity in the corresponding peptide-coated wells and the mean of the difference defined as Δcpm. When the results of IFA and IRMA were compared by a nonparametric method (Spearman rank correlation), the r_s was 0.87 ($P < 0.001$).

ωSerum samples were incubated with 50 μg $(NANP)_3$ per milliliter for 2 hours at room temperature before performing IFA. ND not done.

♂Serum from G.Z., a human volunteer vaccinated with irradiated *P. falciparum* sporozoites and protected against malaria infection. (Reproduced from Zavala et al., 1985).

TABLE II

Neutralization of the infectivity of *P. falciparum* sporozoites.

Rabbit Immunoglobulin

Origin	Concentration (μ g/ml)	% Inhibition *
anti $(NANP)_3$	100	74.2
	20	95.0
	2	51.3
	1	19.0
	0.1	2.7

*Calculated as 100 − [(mean experimental values/mean of controls) x 100].
(Adapted from reference Zavala et al., 1985).

Even if sterile immunity is not achieved, a large decrease in parasite load could be also beneficial by diminishing the severity of the disease, and perhaps also affecting indirectly the rates of transmission.

Several important questions remain to be answered, such as, for example, what is the function of the repeats? Some colleagues, impressed by their strong immunogenicity, proposed that the repeats are a lure to deviate the immune response of the host from other areas of the CS molecules, or from other molecules more essential for the parasite's survival in the host. Although

this may seem an attractive idea, it does not make sense because Fab fragments of antibodies against the repeats neutralize parasite infectivity. Our working hypothesis is that the repeats are involved in the initial interaction between the parasite surface and the putative hepatocyte receptors. Repeats are present in very high concentration on the parasite surface, since the parasite is covered with CS protein, and every molecule has many repeats. Therefore the probability of a fruitful high avidity encounter with a hepatocyte receptor is enhanced enormously even if the binding affinity of each subunit is very low. Multiple cooperative interactions would be favored if the membrane receptor is mobile, and if the receptor itself is multivalent. In addition, repeats are redundantly expressed within the same polypeptide chain. Therefore mutations in one of the subunits will be of little consequence for the parasite, unless the others are equally affected. This will guarantee that an essential structure for the parasite survival will not be subjected to the vagaries of mutational events. If this hypothesis is correct, we predict that other recognition molecules found on the surface of intracellular parasites will contain internal repeats.

How can we reconcile this hypothesis with the fact that repeats are very immunogenic? Why should a structure so essential for the parasite's survival be so easily recognized by the immune response of the host? This may be explained by invoking a phenomenon well known to parasitologists, that is, concomitant immunity. Indeed, the immune response to sporozoites is not harmful to the blood stages of the malaria parasites because these do not express CS proteins. It can be argued, therefore, that the chances of survival for the host (and also for the parasite) are increased if superinfection is prevented, that is if most invading sporozoites are destroyed.

Sequence of repeats of CS proteins

Species	(Strain)	Sequence
P. falciparum		$\overset{V\ D}{N}ANP$
P. vivax		$\overset{D}{\underset{A}{}}GQPAGDRA$
P. knowlesi	(Nuri)	$EQPAAGAG\overset{R}{G}G$
P. knowlesi	(H)	AGQPQAQGDGAN
P. cynomolgi	(Gombak)	$\overset{G}{D}GAAAAGGGGN$
Amino acids used :		P, N, Q, A, G, D, (E), (V), (R)

Fig. 5: Sequence of amino acids of the tandem repeats found in CS proteins of various malaria parasites.

A second question which remains to be clarified is the molecular mechanisms involved in the evolution of the repeats, some of which are shown in Fig. 5. The difficulty lies in the paradoxical finding that the sequence of amino acids of the repeats is very conserved *within* the CS protein domain but it is much less conserved than the rest of the molecule when CS proteins from various species, or even in some instances, strains of malaria parasites are compared. For example, in the CS genes of various strains (or subspecies) of *P. cynomolgi*, there is extensive DNA sequence homology outside the repeat domain, but the repeats differ widely in sequence and encode antigenically unrelated peptides. Antibodies to the repetitive domain are usually species-specific, but antibodies to some other selected areas of the CS protein react with sporozoites from various plasmodial species. These findings suggest that the repeat domain is subject to stronger selective pressures than the rest of the molecule, most likely originating from the immune response of the host.

It is difficult to envisage, however, how the substitution of one set of repeats by another set is accomplished. A complicating feature is that although within a given CS protein the repeats are remarkably conserved, focal changes do occur. One of the most striking was observed in *P. falciparum*, in which NVDP instead of NANP appear four times irregularly dispersed within the repeat domain. This is hard to explain by independent mutational events. Perhaps intragenic con-

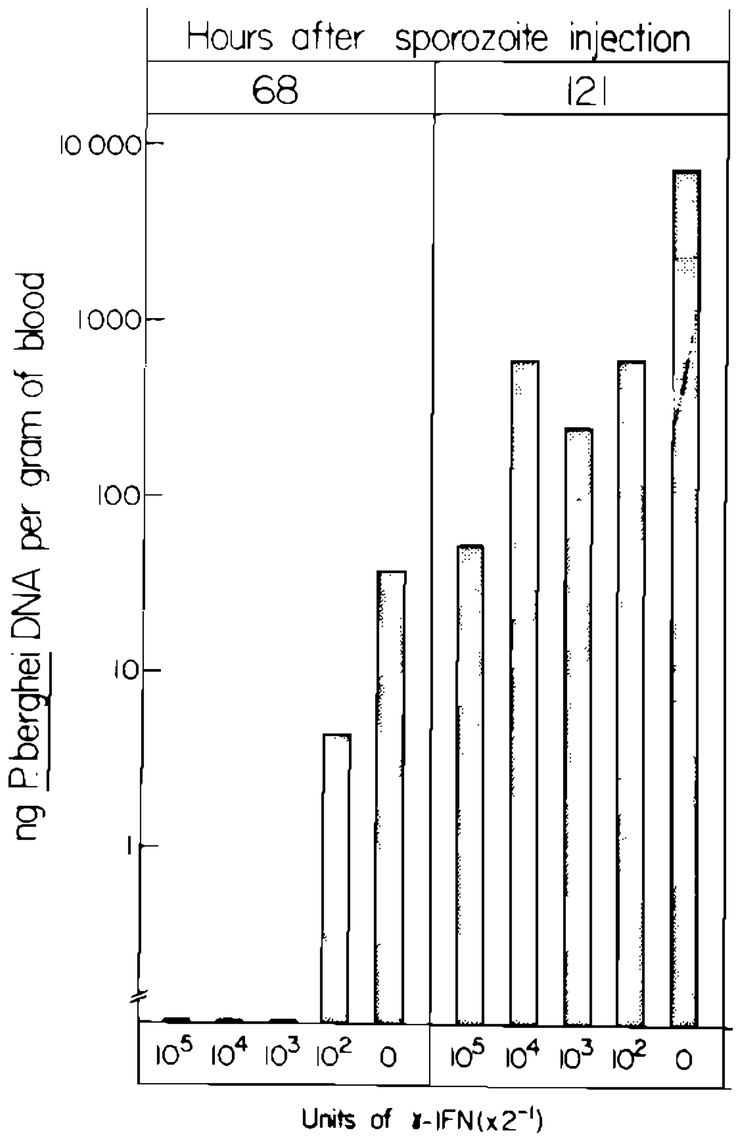


Fig. 6: Parasitemia in mice pretreated with recombinant murine γ-IFN and infected with *P. berghei* sporozoites. The amounts of *P. berghei* DNA in the blood were calculated from the results of DNA hybridization experiments by comparing the values obtained for the animals pretreated with γ-IFN with those obtained in animals injected with PBS. (Adapted from Ferreira et al., 1986).

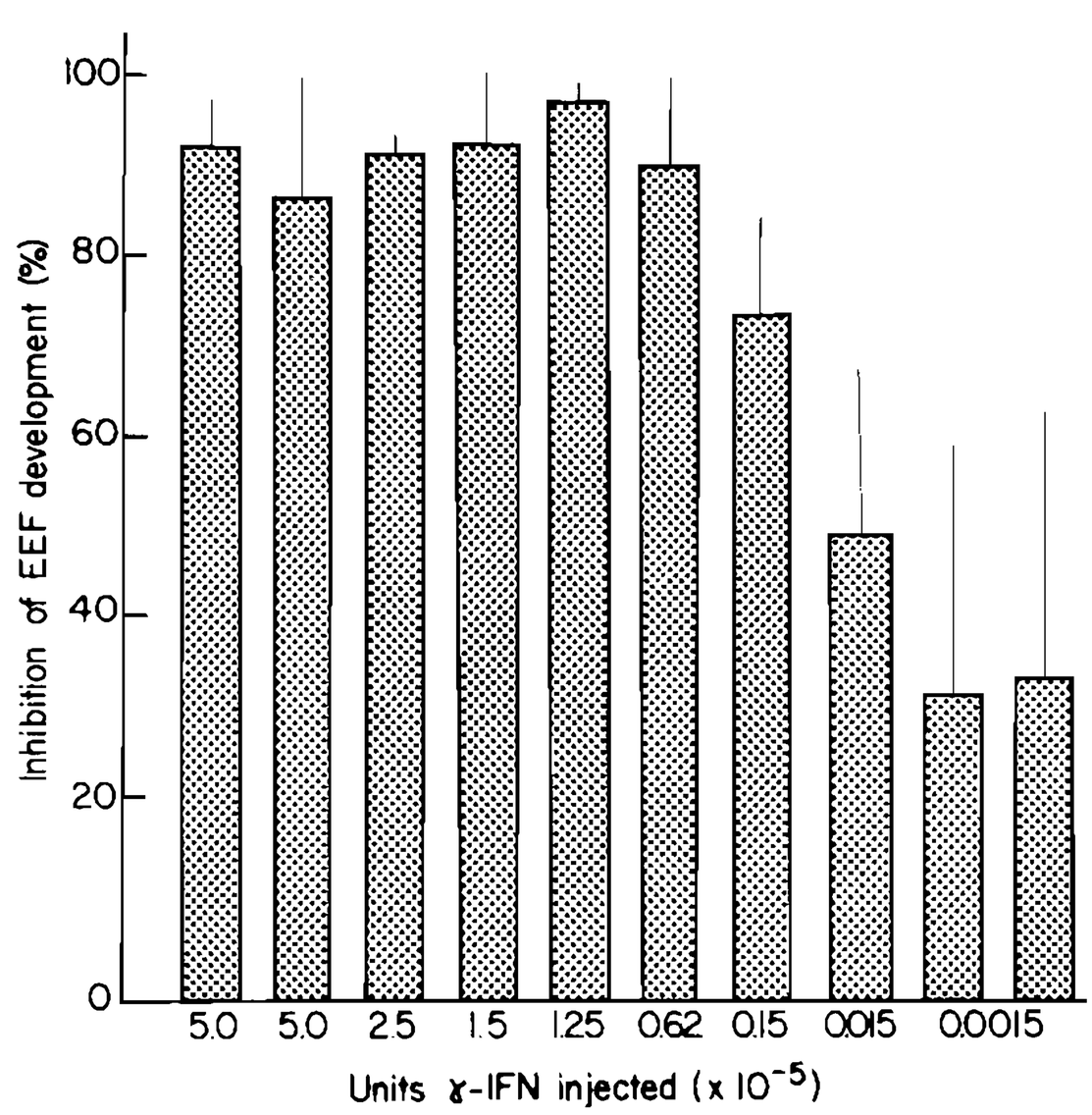


Fig. 7: Inhibition of the development of *P. berghei* EEF forms by recombinant rat γ-IFN. Groups of four to six female Norway Brown rats (Charles River), 2 – 4 months old, were injected intravenously (i.v.) with different doses of rat γ-IFN five hours before the sporozoites. (Adapted from Ferreira et al., 1986). Forty-four hours after sporozoite challenge the rats were bled from the axillary vein and artery, and the livers removed. At this time the development of EEF in the liver is maximal. The DNA was purified from the liver and probed for the presence of *P. berghei* DNA. The amount of parasite DNA per liver was calculated with respect to a standard curve. Values, expressed as means and standard deviations, represent the percent inhibition of EEF development and were obtained by comparing the amounts of hepatic parasite DNA present in γ-IFN and in control groups.

version takes place following changes in one repeat subunit. It is not known whether the variant subunits derive from each other by gradual changes. Instead they may originate from nucleotide sequences selected in evolution, located elsewhere in the genome, and which are rearranged to form new functional subunits.

There are also obvious limitations in the types of amino acids which can be part of the repeat subunits (G, A, P, N, D, Q, and only rarely R, V, E). This suggests that there are essential constraints in the structural features of the repeat domain. This idea is supported by the findings that within each domain the amino acid sequence of the tandem repeats is more conserved than the corresponding nucleotide sequence. Also, as mentioned earlier, in all CS proteins the repeat domain may consist of a series of reverse turns. Perhaps the repeats from neighboring CS molecules interlock to form a sheath surrounding the parasite. Variations in the structure of the repeat subunits which interfere with the assembly of the required quaternary structure may be lethal for the parasite.

Whatever the genit mechanisms involved, variation in the repeats is a potential problem for the immunoprophylaxis of malaria. If under selective pressure of the immune system, populations of sporozoites can rapidly change the CS protein repeats, the effectiveness of repeat-containing vaccines will be limited. The available evidence, however, does not justify a pessimistic outlook. As mentioned already, CS proteins from all isolates of *P. falciparum* and *P. vivax* obtained from Latin America, Asia and Africa contain the repetitive epitope recognized by the same monoclonal antibodies. Furthermore, CS proteins from *P. falciparum* from Thailand and Brazil have been sequenced and they contain $(NANP)_3$ repeats. Relatively high levels of antibodies to this epitope are found in the endemic areas in the serum of adults, who after prolonged exposure have developed resistance to malaria infection. It appears, therefore, that genetic changes leading to the entire substitution of the repeats of the human malaria CS proteins are either rare or compromise the viability or infectivity of sporozoites.

In the last part of my lecture I will present data on the effect of γ-interferon (γ-IFN) on the exoerythrocytic forms (EEF) of malaria parasites.

Because infected hepatocytes do not display parasite-derived or other neoantigens on their membranes, the EEF are protected from direct attack by antibodies. Also, there is no accumulation of inflammatory cells around EEF. It has been previously shown, however, that interferon inducers, or partially purified preparations of interferons, reduce the severity of sporozoite-induced, *P. berghei* infection in mice. Drs. Ferreira and Schofield have used the specific DNA probe isolated by Dr. Enea to study the effect of recombinant rat, mouse and human γ-IFN on the course of sporozoite-induced malaria infections.

In the initial series of experiments, Dr. Ferreira injected groups of five A/J mice intravenously with doses of 200 to $2x10^5$ units of mouse recombinant γ-IFN, five hours before challenge with $5x10^3$ *P. berghei* sporozoites. Blood samples were assayed for the presence of parasite DNA using the ^{32}P-labeled repetitive DNA probe isolated by Dr. Enea. Although all treated animals had parasites in the blood on the sixth day, the prepatent periods were greatly prolonged and parasitemias were 90 to 99% lower than in the controls, even when the injected dose of γ-IFN was only 200 units (Fig. 6).

In contrast, the injection of large daily doses of γ-IFN ($2x10^5$ units/day) had no effect on the parasitemia if the treatment started 72 hours after sporozoite challenge, at a time when the parasites had left the liver and already entered the red blood cells. These findings suggested that the effect of γ-IFN was exclusively on the EEF. To demonstrate this point, Dr. Ferreira used as experimental animals juvenile Norway Brown rats, because a larger proportion of *P. berghei* sporozoites penetrate hepatocytes and develop into EEF in these animals. This permitted direct measurement of the effects of γ-IFN on the EEF, even though the amount of parasite DNA was only a minor fraction of the total liver DNA. The amount of parasite DNA was measured in the livers at 44 hours after challenge, when EEF development was maximal.

Again, the γ-IFN treatment had a striking inhibitory effect on the EEF development when administered five hours before the sporozoites. In two separate experiments, 150 units of interferon (10-20ng protein) were sufficient to inhibit approximately 30% of EEF development, measured at 44 hours following challenge. Close to 90% of inhibition was reached with $6x10^4$ units of γ-IFN (Fig. 7). The effect of the treatment was reversed by preincubation with specific antibodies to rat γ-IFN. The inhibitory activity on the EEF was much less marked if rat γ-IFN was injected 18 hours before, or after sporozoite challenge.

These results encouraged us to test the effect of γ-IFN on EEF of *P. vivax*, one of the most important causative agents of human malaria. The experiments were performed with human γ-IFN in four chimpanzees, an animal species which is susceptible to infection with *P. vivax* spo-

rozoites. Two chimpanzees received daily doses of $5x10^6$ units of interferon for six days, starting five hours before they were challenged with a relatively large number (10^5) of sporozoites of the Chesson strain of *P. vivax* given to us by Dr. W. Collins. On day seven, all chimpanzees were splenectomized to permit the development of the blood stages. Blood smears were examined for the presence of parasites, starting at day 8. As shown in Fig. 8, the prepatent periods in the control animals were 10 and 12 days, and in the experimental animals 12 and 16 days. The levels of parasitemia were much reduced in the γ-IFN-treated animals until day 16, when curative treatment with chloroquine and primaquine was started.

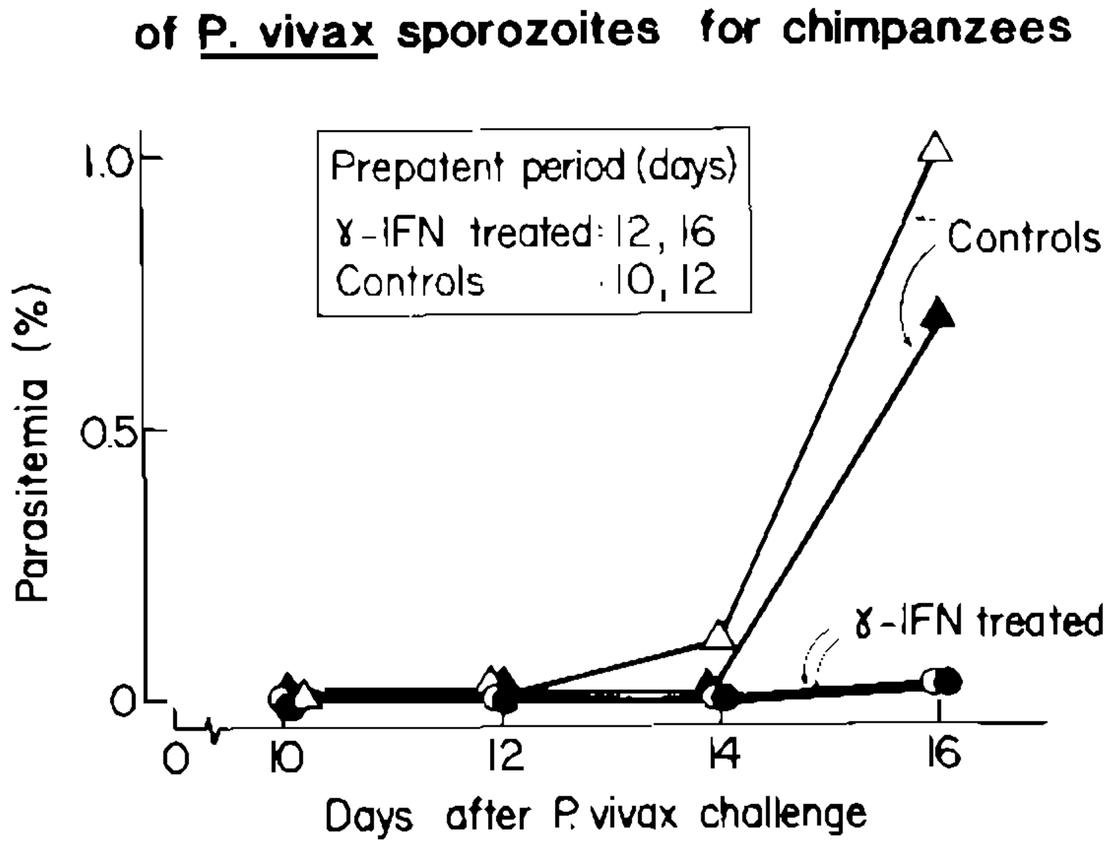


Fig. 8: Inhibition of *P. vivax* infectivity in chimpanzees pretreated with recombinant human γ-IFN. Two chimpanzees were injected i.v. with 5×10^6 units of γ-IFN (diluted in PBS) at minus 5 hours, plus 2 hours and daily on days 1 through 6; the other two received PBS injections. At time 0, the animals were challenged with 10^5 *P. vivax* sporozoites, freshly dissected from the salivary glands of *Anopheles stephensi* mosquitoes. On day 7, the animals were splenectomized to enhance the development of the blood stage parasites. Two blood smears per animal were prepared starting on day 8 and every other day thereafter. A total of 2×10^4 red blood cells was counted in two slides. Results are expressed as percent parasitemia. (Reproduced from Ferreira et al., 1986).

Finally Dr. Schofield investigated the effect of recombinant human γ-IFN on the development of *P. berghei* in an *in vitro* system, using a human hepatoma cell line. Pretreatment of the target cells, 24 hours prior to sporozoite invasion, with recombinant human γ-IFN at concentrations greater than 1 unit/ml, totally inhibited the multiplication of parasites. In cells exposed to 1 unit/ml γ-IFN, EEF multiplication was reduced by 92%, as compared to untreated controls ($p < 0.0001$ by one-tailed t test).

In short, γ-IFN had a profound effect on the *in vitro* and *in vivo* development of EEF. Its inhibitory activity *in vivo* could be measured accurately using a specific DNA probe, because EEF are found exclusively in the host's liver cells. Maximal (but not total) inhibition of EEF development was achieved with $0.62x10^5$ units, but significant effects were observed with doses as low as 150 units (~15ng protein). The activity of γ-IFN was greatest when injected a few hours before challenge with sporozoites. This is in contrast to prior observations that interferon inducers (e.g., Newcastle disease virus) were most effective in protecting mice against malaria if given twenty hours after sporozoite inoculation. The effects seen with interferon inducers were probably mediated by α- and β-interferons, which may inhibit EEF by different metabolic pathways.

The mechanism by which γ-IFN inhibits EEF development is unknown. γ-IFN activates oxidative metabolism in macrophages, as well as increases their antimicrobial activity. In addition, γ-IFN can destroy parasites by respiratory burst-independent mechanisms, since it prevents the *in vitro* growth in fibroblasts of some microorganisms such as, for example, *Toxoplasma gondii*. Al-

though it cannot be excluded that macrophages or natural killer cells recognize the few randomly distributed infected hepatocytes and destroy the parasites, this seems improbable. Instead, the targets of the lymphokine may be the hepatocytes themselves. Indeed, Dr. Schofield showed here that γ-IFN at doses equal to or lower than those required for its antiviral activity, prevented the growth of EEF inside a hepatoma cell line *in vitro*. We speculate that γ-IFN, either directly and/or through a lymphokine "cascade", may render the liver cells unsuitable for the development of the malaria parasite.

Are these observations of relevance to natural or acquired immunity to sporozoites? I remind you that Dr. Ojo-Amaize observed that the spleen cells of mice immunized with irradiated sporozoites released high levels of γ-IFN when challenged *in vitro* with antigen, and that interferon can be detected in the serum of *P. falciparum*-infected patients. It seems plausible therefore that interferons play a role in the protective immunity to malaria conferred by vaccination with irradiated sporozoites or by repeated exposure to infected mosquitoes in endemic areas. From the practical point of view, it is well known that some EEF of *P. vivax* and *P. ovale* remain viable in the human liver for long periods of time after the elimination of the blood stages. It would be of interest to determine whether recombinant interferons destroy these dormant forms of the parasite and prevent clinical relapses.

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