CHAPTER V

DISCUSSION

1. General Remarks about Pulse Field Gradient Gel Electrophoresis of *Plasmodium* Chromosome

The separations were quite sensitive to a variety of experimental variables such as the effect of agarose and temperature (Mathew et al., 1988a), effect of pulse time and electric field strength (Mathew et al., 1988b) , effect of electric field shape (Cantor et al., 1988) and DNA topology (Mathew et al., 1988c). In this study, the pulse times, voltages, and electric field configurations were varied to display the karyotypes of *P. falciparum*. In conclusion, the suitable running conditions to show all chromosomes within a gel in this experiment were 80 volt for 137 hr at 14°c with pulse time ramped 180-900 sec (figure 10.11).

However, the conditions chosen depend on the size range of the chromosomes which are of particular interest for the research being done. The conditions that permit the resolution of only the smallest or the largest chromosome which are in a compression zone may not be suitable for overall karyotype comparisons, as shown in figure 10.1-10.5.

Compared to the gel which was fractionated by OFAGE technique (figure 10.1) to the other gels, it was found that the resolution of PFGE was dramatically affected by the configuration of the electrode used. This is because the shape of electric fields applied was altered resulting migration in different manner. The hexagonal electrode was proved to be the most suitable, and yielded an angle of 120 degrees between the alternate electric fields. This observation was in agreement with earlier studies (Chu et al., 1986; Cantor et al., 1988). Small changes in the configuration of PFGE equipment and in buffer and voltage conditions can radically affect the resolution of the large DNA fragments (Chu et al., 1986; Carle and Olson, 1987).

At present, the number of chromosomes in P. falciparum is 14 (Wellems et al., 1987). Unlike this report, only 11 chromosomes in P. falciparum were demonstrated with the ramped pulse time from 180 - 900 sec, 80 volt for 137 hr in this study. It is possible that some chromosomes still remained at the slot or they were unfractionated e.g. high intensities of ethidium bromide staining at the area of chromosomes 5, 6, and 7 were observed.

Since artifacts in PFGE can occur, especially for the largest DNA fragments (Carle and Olson, 1987), proof of the chromosome number of P. falciparum chromosomes should probably involve the reproducible and good resolution of the DNA

bands on pulsed field gels. In order to prove whether a single band of chromosome observed on an ethidium bromide - stained gel represents a single or more than one chromosome, each of the bands of special interest can then be cut out from the gel and subjected to rare-cutting restriction enzymes. These chromosomal DNA fragments will be fractionated again by PFGE. After separation, they will be southern blotted and hybridized with a probe (eg. telomeric probe). The number of the fragments can be counted and thus the number of chromosomes in one band could be identified (Janse, C., personal communication).

2. Chromosome Size Polymorphism in Plasmodium

The parasites from Thailand used in this experiment were pyrimethamine-sensitive and resistant clones grown under controlled laboratory conditions. They were carefully examined for a characteristic of susceptibility to pyrimethamine. The parasites from China and Vietnam were shown to be naturally resistant to pyrimethamine .

The results described above showed some differences of chromosome patterns in Thai sensitive and mutant clones, in Chinese isolate and its clones, and in the Vietnamese clones. These changes in chromosome structure might reflect an adaptation of these parasites to cell culture rather than a real genetic polymorphism of parasites in the wild. Because the

parasites in these studies were confined to the erythrocytic stage of the life cycle. The effect of sexual recombination, which occurs in mosquitoes, was not studied in these experiments.

The change of chromosome sizes in clonal level has been considered significant. The present demonstration of changes in clone T9/94 RC1-RC4 appeared to be strange because these clones were derived from the T9/94 clone by micromanipulation and were still as sensitive as the parent clone with the pyrimethamine MIC of $5x10^{-8}$. It is implied that long-term in vitro cultivation may reflect generalized modifications of genomic structure of T9/94, thus resulting in chromosome polymorphisms of its subclones. This notion is in agreement with the previous evidence that P. falciparum can lose genetic information, especially during long-term culture in vitro (Pologe and Ravetch, 1986; Corcoran et al., 1986). Consequently, it is not surprising that a remarkable diversity in chromosome size from isolate to isolate was detected (Babiker et al., 1991) because it is evident in this study that chromosome heterogeneity occurred even in the subclones that were still unchanged in regard to drug sensitivity.

The mutant T9/94 S300/300 clone was considerably different from T9/94 in chromosome 9. Though the other mutant clones, T9 /94 (M1-1) a1, T9/94 (M1-1) a2, T9/94 (M1-1) a6, T9 /94 (M1-1) b1,T9/94 (M1-1) b5, T9/94 (M1-1)b9, had the same pyrimethamine MIC ($1x10^{-6}$), their ninth chromosomes were

slightly different from those of the parent clone. One supposition that could account for this extensive chromosomal polymorphism was the different kind of mutagens used. Both EMS (ethylmethane sulfonate) and MNNG (N-methyl-N'-nitroso-N-nitroguanidine) are the alkylating agents (Mays, 1981). They may introduce alkyl group into nucleotides at various positions, thus giving the different mispairing. As a result, the parasite may change the DNA structure in response to environmental conditions.

Likewise, chromosome 9 from mutant T9/94 (M1-1) b1-b14 clones was also somewhat larger than T9/94. That the mutant clones were different from parent in chromosome 9 therefore implied that mutations be produced by mutagens utilized in cultures and both alkylating agents affected the ninth chromosome of P. falciparum. This is consistent with the parallel evidence that chromosome size variations of P. falciparum by PFGE were more numerous in parasite populations made mefloquine resistant by cultivation under drug pressure than in control parasites maintained in culture medium alone (Wellems et al.,1988).

Extensive variation in another 4 Thai clones from isolate K31 was observed by having 3 chromosome patterns. In addition, even though their drug susceptibility were identical to that of T9/94,the karyotypes were different from T9/94 and its derivatives.

For the parasites from China and Vietnam, no two identical clones was obtained and the clones were different from their isolate. The chromosome patterns of these parasites were also different from those of the T9/94 clone. These differences may be due to the geographic distribution of this isolate. This is because the most marked difference was in the large chromosome from chromosome 9 to 11 not in chromosome 4. Another implication is that chromosome 10 and 11 should certainly not involved the pyrimethamine resistance. This results from that these 2 chromosomes in all Thai clones were larger than those of Chinese parasites which were more resistant to pyrimethamine but smaller than those of Vietnamese clones which were the most resistant clones in this experiment. If the tenth and eleventh chromosomes were correlated with pyrimethamine resistance, the chromosome size would change in the parallel way, that is either smaller or larger in both Chinese and Vietnamese parasites. Both chromosomes should be further analysed.

Though the mutant clones [T9 /94 (M1-1) a1, b1, b5, b9, and T9/94 S300/300] had the same pyrimethamine MIC ($1x10^{-6}$),PFGE can reveal the chromosome 7 and chromosome 9 - sized polymorphisms. Therefore, the PFGE technique could be potential for characterization of P. falciparum parasites.

3. Chromosome 4 Polymorphism

A striking phenomenon was the larger size of chromosome 4 ofT9/94 (M1-1) b6 and T9/94 (M1-1) b14 which were the most resistant to pyrimethamine amongst the mutant clones tested here, showing an MIC value of $5x10^{-8}$. Combined with the result that DHFR-TS probe bound to chromosome 4 of T9/94 and the Vietnamese clones, it is likely that there was a relationship between this alteration of the fourth chromosome and pyrimethamine resistance. To confirm this assumption, further investigation for the fourth chromosome of both T9/94 (M1-1) b6 and T9/94 (M1-1) b14 clones is required.

There was no chromosome 4 size polymorphism in the T9/94 (M1-1) a1, T9/94 (M1-1) a2, T9/94 (M1-1) a6, and T9/94 (M1-1) a9 group. They might be cloned from the same parasite because they also showed no differences for the other characteristics such as enzymes types, protein patterns (Thaithong, S., unpublish data). Thus, the chromosome patterns of these few clones by PFGE were correlated to the data from another method of malaria characterization.

Again, it is indicated that PFGE technique could be exploited for characterization of P. falciparum parasites.

No convincing indication from karyotypes generated by PFGE in this study elucidates that pyrimethamine resistance is due to DHFR amplification because of the faulty filter membranes.

However, it is likely that DHFR genetic material is approximately located on the fourth chromosome when comparing the bands from ethidium bromide-stained gel with those from autoradiogram. This is in agreement with the previous reports (Tanaka et al., 1990; Cowman et al., 1988).

As there is a possibility that amplification of genes may contributed to the chromosome size polymorphism described above, it would be interesting to examine this hypothesis. This was done and discussed below in dot blot analysis.

4. Dot Blot Analysis

The molecular basis of parasite resistance to pyrimethamine is not well understood and has given rise to several interesting controversies (Babiker et al., 1991).

A separate study of all parasites investigated by PFGE analysis were examined by dot blot analysis. Unfortunately, this work could not be completed because of the faulty Hybond-N membrane. After using Hybond-C membrane, another 2 pyrimethamine-sensitive and pyrimethamine-resistant clones of P. falciparum were successfully compared.

From a hypothesis that the mechanism of pyrimethamine resistance of P. falciparum may be due to DHFR-TS gene amplification, one nitrocellulose membrane was probed with the radiolabelled DHFR-TS gene. If the gene in the resistant clones

had been amplified, more DHFR copies in their DNA would have been detected than in the DNA of the sensitive clone. This is because equal amounts of DNA from each parasite were spotted on the membrane. Different intensities of the signal, however, may be due to the RNA contamination in crude DNA preperations, resulting in the different quantities of total DNA remaining in the each spot (albeit RNA in the crude extracts cannot interfere the result of DHFR probing because it was degraded by NaOH solution).

To confirm that DNA was equally loaded in this experiment, the other identical dot-blot membrane was prepared and probed with ß-tubulin gene. Not only could the ß-tubulin gene be used as a DNA qualitative control but also imply the quantity of copies of DHFR-TS gene as a consequense of the report that there was a constant single ß-tubulin gene in P. falciparum (Holloway et al., 1990).

Compared to ß-tubulin gene, another gene , rep 20 sequences, occurred only in P. falciparum and could thus provide a species-specific probe. However, hybridization patterns in this parasite probed with rep 20 were highly variable. Moreover, these sequences resided on all chromosomes of P. falciparum separated by OFAGE (Oquendo et al., 1986). In contrast to the other report , some clones of P. falciparum were devoid of rep 20 on one or both ends of chromosomes 1 or 2 and it is also evident that blocks of rep 20

varied in copy number (Corcoran et al., 1988). By this criteria, rep 20 sequences should not be served as a good control probe in this study. When the digests of DNA extracted from 4 clones of P. falciparum was probed with ribosomal RNA fragments (λPfrib2), the same hybridization patterns were demonstrated (Oquendo et al., 1986). It is obvious from copy analysis that each of the 4.4 kb and 4.2 kb rRNA fragments of P. falciparum was presented approximately 4 times per haploid genome, and that there was a total of only 8 rRNA genes (Langsley et al., 1983). Ribosomal RNA gene could also be a control DNA probe by its constant copies number. As mentioned above, nevertheless, only one β-tubulin gene was found in P. falciparum, thereby resulting in easier interpretation than rRNA probe.

Furthermore, intensity differences may be discovered owing to an insufficient amounts of probe. Thus, equivalent volumes of these serially diluted DNA were dot blotted in duplicate onto each membrane to ensure the reproducible result.

The radioactivity on the fiters quantitated by liquid scintillation counter revealed that no amplification in the pyrimethamine-resistant clones occured. This is in contrast to the previous evidences. Some investigators have indicated that an increase in pyrimethamine resistance of P. falciparum (Inselburg et al., 1987) or P. chabaudi (Cowman and Lew, 1989) may involve duplication of the DHFR gene. However, an early report by Inselburg et al. (1987) was later shown that

an augmentation in pyrimethamine resistance was caused by a contamination of another clone (Tanaka et al., 1990). On the other hand, my result is consistent with Chan et al. (1989) that the resistant (HB3) clone had the same number copies of DHFR gene as sensitive (3D7) one. Also, it confirms an earlier study (Snewin et al., 1989) that the high level of resistance in P. falciparum is not due to gene amplification and there is a single copy of DHFR- TS gene in both pyrimethamine-sensitive and pyrimethamine-resistant parasites.