A Developmental Defect in *Plasmodium falciparum* Male Gametogenesis

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Abstract. Asexually replicating populations of *Plasmodium* parasites, including those from cloned lines, generate both male and female gametes to complete the malaria life cycle through the mosquito. The generation of these sexual forms begins with the induction of gametocytes from haploid asexual stage parasites in the blood of the vertebrate host. The molecular processes that govern the differentiation and development of the sexual forms are largely unknown. Here we describe a defect that affects the development of competent male gametocytes from a mutant clone of *P. falciparum* (Dd2). Comparison of the Dd2 clone to the predecessor clone from which it was derived (W2'82) shows that the defect is a mutation that arose during the long-term cultivation of asexual stages in vitro. Light and electron microscopic images, and indirect immunofluorescence assays with male-specific anti-α-tubulin II antibodies, indicate a global disruption of male development at the gametocyte level, with at least a 70–90% reduction in the proportion of mature male gametocytes by the Dd2 clone relative to W2'82. A high prevalence of abnormal gametocyte forms, frequently containing multiple and unusually large vacuoles, is associated with the defect. The reduced production of mature male gametocytes may reflect a problem in processes that commit a gametocyte to male development or a progressive attrition of viable male gametocytes during maturation. The defect is genetically linked to an almost complete absence of male gamete production and of infectivity to mosquitoes. This is the first sex-specific developmental mutation identified and characterized in *Plasmodium.*

In the course of its life cycle between mosquito and man, the human malaria parasite *Plasmodium falciparum* passes through several developmental changes, among which is the obligate development of sexual forms from asexually replicating stages. This process starts in the human red blood cells when individual asexual parasites, triggered by a poorly understood process, stop dividing and differentiate into gametocytes. After a 1–2-wk period of development through distinct morphological stages, micro- (male) and macro- (female) gametocytes mature so that, when taken up into the mosquito midgut, they are able to transform into male and female gametes free from the red blood cells.

The production of male gametes, a process known as exflagellation, is a spectacular event easily observed by light microscopy. Within 10–20 min, dramatic nuclear and cytoplasmic reorganization events lead to the release of eight male gametes from an individual male gametocyte. During individualization of these gametes, emerging flagella attach to nearby red blood cells and form animated clusters termed exflagellation centers. Each female gametocyte, by contrast, gives rise to a single, large gamete that appears round and motionless by light microscopy. Gamete fusion in the mosquito midgut may occur between male and female gametes from the same parasite line (self-fertilization) or may involve different lines (cross-fertilization). A tremendous parasite diversity, challenging the use of agents aimed at controlling the disease, can therefore be generated through the meiotic recombination activity following fertilization (2, 13). Individual fertilization events lead to single oocysts that set up in the midgut wall and give rise to thousands of haploid sporozoites capable of migration to the mosquito salivary glands and infection of the next human host.

A clonal population of haploid parasites can generate both male and female gametocytes. In this homothallic heterogametic system, the transformation into sexual stages implies two types of developmental decisions: switch to sexual differentiation and commitment to male or female determination. The male and female pathways, leading to morphologically and functionally distinct forms, must then involve differential expression of sex-specific

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genes. The molecular events of sex determination and differentiation remain to be discovered, and in particular it is not known how the commitment to the sexual pathway and to male or female development are related. A few sexual stage–specific proteins have been identified (3, 19, 29), most of them being expressed by both sexes, but they are usually assessed as potential transmission-blocking vaccine candidates and their biological function is generally not known. A few mutant lines that do not produce gametocytes have also been reported in which sexual development appears to be blocked in early stages (6, 21, 26). Although some gametocyte production failures have been associated with a deletion from chromosome 9 of *P. falciparum* (1, 6), searches for sexual stage genetic determinants have been generally hampered by the difficulty of conducting genetic analysis in *Plasmodium.* Indeed, only two laboratory genetic crosses in *P. falciparum* have been performed (40, 42).

In previous studies, we showed that one parent of a *P. falciparum* cross (Dd2) infected mosquitoes poorly on its own (39), and this defect was traced to a reduced production of exflagellation centers, indicating poor formation of male gametes (38). The defect was inherited as a single Mendelian trait that mapped to the parasite's twelfth chromosome (38). In this report, we show that the mutation arose during continuous in vitro cultivation of a *P. falciparum* clone (W2'82) under mefloquine pressure, and that W2'82 and its subclone Dd2 therefore constitute an isogenic pair suitable for cytological comparisons and genetic studies. Comparisons of Dd2 and W2'82 gametocytogenesis in red blood cells have revealed a developmental defect in Dd2 that results in severely reduced production of mature male gametocytes relative to female gametocytes. This defect and the poor mosquito infectivity are genetically linked. Morphologic comparisons show that abnormal gametocyte forms are frequently associated with this defect. These observations suggest a genetic mutation in pathways that determine or support the differentiation and development of male gametocytes.

Materials and Methods

Parasites

P. falciparum 3D7 (40), W2'82 (24), and Dd2 (42) clones were maintained in vitro by standard methods (36) using O+ human erythrocytes. In vitro induction of gametocytes was as previously described (15) from parasites that had been cultivated from cryopreserved stocks for <3 wk. Gametocyte development was followed by examination of Giemsa-stained smears of infected cells. Gametocytes were usually first apparent 5–7 d after induction.

Feeding to Mosquitoes

Functional maturity of stage V gametocytes was assayed by light-microscopic observation of emergence and exflagellation from induced cultures. For these in vitro assays, the gametocyte-infected red blood cells were pelleted by centrifugation, resuspended at 50% hematocrit in heat-inactivated human serum, and incubated at room temperature for 10–15 min. On the day of maximum exflagellation (1–2 d after the first exflagellation center was observed) and on the day thereafter, the gametocytes were used to infect *Anopheles freeborni* mosquitoes by membrane feeding. Preparation of gametocytes and membrane feeding were performed as described (30, 39). Gametocyte concentrations were adjusted to 1% before feeding. Oocyst counts were determined 7 d after feeding, from mercurochrome-stained midguts of mosquitoes that contained eggs and therefore had engorged.

Giemsa Staining and Relative Counts of Male and Female Gametocytes

A good discrimination between male and female mature gametocytes was typically obtained from methanol-fixed blood films incubated for 30 min in a 1:15 dilution of modified Giemsa stock (supplied in pH 6.9 buffer, Sigma Chemical Co., St. Louis, MO) and deionized water (pH 5.5–6.0). Slight modifications of stain concentration and incubation time were sometimes used on the basis that, in the blue–red balance, red color tended to be enhanced by lower stain concentration and longer incubation.

In counting gametocyte sex ratios, we took into account only normal-looking stage V male and female gametocytes. Criteria for normality and stage assignments were based on shape of the parasite, color and texture of the cytoplasm, and aspect and organization of the nuclear area (4). Dd2 and W2'82 gametocytes were always examined side-by-side at each step of their development. For each count, the sexes of at least 100 gametocytes were recorded and, for most counts, >400 were recorded. For genetic linkage analysis, sex ratios were determined from slides of nine independent progeny clones from the HB3 × Dd2 cross (38). Statistical significance of the sex ratios was determined by application of the *t* test to the logarithms of sex ratios in the two parental groups.

Propidium Iodide Staining

Giemsa-stained slides were rinsed in distilled water, air dried, fixed for 10 min in Carnoy's solution (6:3:1 ratio of ethanol/chloroform/acetic acid), rinsed in distilled water, air dried again, and stained under coverglass with propidium iodide 4 μg/ml for >30 min at room temperature.

Immunostaining with Anti–α-tubulin II and Anti-Pfs230 Antibodies

Primary antibodies were a rabbit serum against a COOH-terminal synthetic peptide sequence (NH_2-CEGEDEGYEY-COOH) of *P. falciparum* α-tubulin II (Fried, M., and D.C. Kaslow, manuscript in preparation) and a mouse serum against the fusion protein maltose-binding protein (MBP)+Pfs230–derived r2 peptide (31). Serum of rabbits immunized with Keyhole Limpet hemocyanin (KLH) and anti-MBP mouse serum were used as controls, respectively. Secondary antibodies were TRITC-conjugated goat anti–rabbit IgG and FITC-conjugated goat anti–mouse IgG (Southern Biotechnology Associates Inc., Birmingham, AL). Parasites in culture medium were air dried on toxoplasmosis slides (No. 5638-01940; Bellco Glass, Inc., Vineland, NJ) and fixed and stained as described (29) except that the blocking buffer composition was 0.05% Triton X-100 and 5% goat serum (No. G-9023; Sigma Chemical Co.) in phosphate-buffered saline, and slides were mounted in SlowFade (Molecular Probes, Inc., Eugene, OR) after staining. Before use, the two primary antibodies were mixed at a final concentration of 1/4,000 each and the two secondary antibodies were mixed at a final concentration of 1/200 each.

Microscopy and Photography

For Figs. 3 and 5, *A–D*, images were photographed with a Zeiss Axiophot microscope (Thornwood, NY) using Kodachrome 25 film (Kodak, Rochester, NY) and a 80A filter (Giemsa-stained samples) or Fuji 1600 film (Tokyo, Japan) and 490 nm (FITC) or 550 nm (TRITC and propidium iodide) illumination. Analyses of fluorescence patterns were from photographs of random fields. For Figs. 2 and 5, *E–G*, Giemsa-stained parasites and immunofluorescence signals were visualized with a Zeiss Axiophot microscope equipped with strain-free optics and a vertical fluorescent illuminator. The Zeiss 487917B1 filter set was used for immunofluorescence involving TRITC and the Zeiss 487915B1 filter set was used for immunofluorescence involving FITC. A custom-built computer-controlled shutter (J.A. Dvorak), a light attenuator, infrared- and heat-blocking filters, and beam-shaping optics were placed in the optical path between the HBO-100 mercury arc lamp and the vertical fluorescent illuminator to minimize radiation-induced fading of fluorescent reactions. Video images were obtained from the microscope with an image intensifier (No. C2400-68; Hamamatsu Photonic Systems, Bridgewater, NJ) and relayed onto the faceplate of a Hamamatsu 2400-60 CCD camera. Magnification of the video image was adjusted with projection optics placed between the mi-

croscope and video camera. The video signal was monitored with an oscilloscope, combined with time and date code, and the composite signal was captured into computer memory with a Data Translation DT2862 video frame grabber (Marlboro, MA) operating in an IBM PC-AT–compatible 80468 computer. The automatic gain controls of all video electronic circuits were disabled. To demonstrate the relationship between rhodamine and FITC reactions, two consecutive images of the same specimen field were collected using the fluorescence filters specific to the reactions. The two resulting 512 × 480 pixel × 8 bit monochrome images were converted into 512 × 480 pixel × 24 bit true-color (TARGA) images. The green and blue channels of the rhodamine image and the red and blue channels of the FITC image were eliminated. The resulting rhodamine "red" image and "green" FITC image were merged in the computer, saved as a composite TARGA file, and transferred to Kodak Ektachrome 100 PLUS Professional film with a Lasergraphics film recorder (Irvine, CA). Colocalization of rhodamine and FITC resulted in the appearance of a secondary yellow color because of the overlapping of primary red and green colors. High-magnification, high-resolution, Giemsa-stained cells were imaged by transmitted light with the microscope described above. A filter wheel containing a red, green, and blue filter was inserted into the optical path. The microscope focus was optimized for each color. Three monochrome images of each specimen were recorded with a video camera (series 68 SIT; Dage-MTI, Inc., Michigan City, IN) and processed to produce color images as described above.

Electron Microscopy

Samples were fixed with 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.4, with 4% sucrose for 2 h and then postfixed in 1% osmium tetroxide for 1 h. After staining with 0.5% uranyl acetate, the materials were dehydrated in ascending concentrations of ethanol and embedded in Epon 812. Ultrathin sections were stained with 2% uranyl acetate in 50% methanol and with lead citrate and examined in a Zeiss CEM902 electron microscope.

DNA Isolation and Fingerprinting

DNA extraction, endonuclease digestion, agarose gel electrophoresis, and filter hybridizations were performed as described (8). Hybridizations were performed at 56°C and blots were washed three times for 10 min at room temperature in 2× standard saline/phosphate/EDTA (SSPE) with 0.5% SDS, followed by two higher stringency washes at 50°C in 0.3 × SSPE with 0.5% SDS.

PCR Analysis of Mosquito Oocysts

Mosquitoes fed 10 d previously with W2'82 mature gametocytes were dissected and their midguts individually collected and ground with a pestle in grinding buffer (0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl, pH 9.2, 0.05 M EDTA, pH 8.0, 0.5% SDS). Proteins were denatured by incubation at 65°C for 30 min, precipitated by incubation on ice for 30 min in a 100:28 ratio of grinding buffer and 7.5 M potassium acetate, and pelleted by centrifugation at 14,000 *g* for 15 min. The DNA contained in the supernatant was precipitated by addition of 2 vol of 100% ethanol at room temperature for 5 min, pelleted by centrifugation at 14,000 *g* for 15 min, and washed once in 80% ethanol and once with 100% ethanol. For each DNA sample, two nested PCR reactions were performed, as previously described (25) with the oligonucleotide pairs DIA3/SP1 and DIA12/SP1 as diagnostic primers. Template DNA prepared from a mixture of six midguts of 3D7-fed mosquitoes was included as a control. The pair DIA3/SP1 gave a positive signal with the pyrimethamine-sensitive 3D7 clone and a negative signal with the pyrimethamine-resistant W2'82 clone, and the pair DIA12/SP1 gave a positive signal with W2'82 but no visible band with 3D7 DNA.

Results

P. falciparum Clones W2'82 and Dd2 Are an Isogenic Pair that Differ in Mosquito Infectivity and Male Gamete Production

The *P. falciparum* Dd2 clone was isolated from the W2MEF line, which had been obtained from the *P. falciparum* clone W2'82 after 18 mo of continuous cultivation in the presence of mefloquine (24) (Fig. 1 *A*). Changes in mefloquine response of the W2MEF line relative to W2'82 were accompanied by certain chromosome size changes, gene amplifications (e.g., *pfmdr1*), and gene deletions (e.g., HRPII) (41, 43). Multiple polymorphic markers, including drug sensitivity, isoenzyme determinations, two-dimensional electrophoretic comparisons of labeled proteins, and RFLP analyses otherwise confirmed that W2'82 and Dd2 are isogenic and ruled out the possibility that Dd2 was derived from another contaminating line (24, 41).

Because W2'82 clone was obtained directly from fresh culture-adapted parasites (Indo III isolate), we suspected it might not harbor the Dd2 male defect, which was likely to have arisen instead during the long process of mefloquine selection and subcloning. Infectivities of W2'82 and Dd2 gametocytes to *A. freeborni* mosquitoes were therefore compared in membrane feeding experiments. Table I presents the results of two such experiments, in which the number of oocysts were counted from mosquito midguts 7 d after feeding. These experiments also included control feedings with gametocytes of *P. falciparum* clone 3D7 (from the NF54 isolate), which is a commonly used laboratory standard for routine infectivity determinations (40). The results of the oocyst counts show that W2'82 yields normal levels of self-fertilization and oocyst production relative to 3D7, in contrast to the almost complete loss of infectivity that characterizes Dd2 gametocytes.

To confirm the genetic background of the W2'82 and Dd2 parasites used in this work, we performed fingerprint analysis with the pC4.H32 probe, which detects clone-specific patterns of interspersed subtelomeric repeats (8). Fig. 1 *B* shows that the patterns from the parasite lines and clones are the same, except for the apparent loss of a subtelomeric fragment in W2MEF and its subclone Dd2 (Fig. 1 *B*, *arrow*). We also used PCR assays to verify that the W2'82 oocysts recovered from the feeding experiments had not been contaminated by 3D7 parasites. Using primers specific for the dihydrofolate reductase gene (25) and oocyst DNA prepared from individual midguts, we tested for the different codons that determine pyrimethamine resistance in W2'82 and pyrimethamine susceptibility in 3D7 parasites. The oocysts were confirmed to have the Asn-108 codon indicative of the W2'82 pyrimethamine-resistant genotype (data not shown). By contrast, control experiments using oocysts pooled from 3D7-infected midguts indicated only the Ser-108 codon of the pyrimethamine-sensitive genotype.

Consistent with our previous findings (38), a marked reduction in the number of exflagellation centers was also observed in the triggered Dd2 gametocyte cultures: examination of entire slides showed very few (maximum four) Dd2 exflagellation centers, much fewer than the one to three exflagellation centers per 40× field typical of the 3D7 control. Exflagellation of mature W2'82 gametocytes was comparable to the 3D7 control, as expected of a clone yielding regular oocyst numbers.

Abnormal Male Gamete Production Is the Result of a Developmental Defect in Dd2 Gametocytes

Diminished numbers of male gametes may result from a

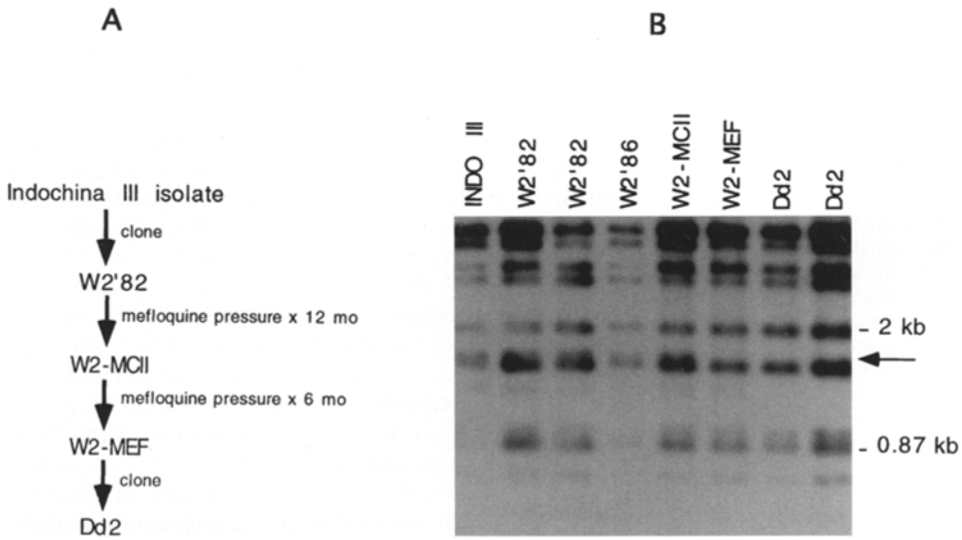


Figure 1. Origin of the Dd2 and W2′82 clones. (*A*) Selection steps in the isolation of clones W2′82 and Dd2 (adapted from [24]). (*B*) DNA fingerprint patterns of lines and clones derived from the Indochina III isolate. HinfI restricted genomic DNAs were blotted and probed with the pC4.H32 probe which gives clone-specific patterns of interspersed repeats. W2′82A and Dd2A represent particular freezer stocks of W2′82 and Dd2 used for gametocyte production in this work. The fingerprint patterns confirm that W2′82 and all of its derivatives originated from a single INDO III parasite. The arrow marks the band deleted from the W2MEF and Dd2 lines.

deficient exflagellation process or from a developmental defect that reduces the numbers of mature gametocytes capable of exflagellation. With the matched pair W2′82 and Dd2, we found that the asexual stages were indistinguishable and that gametocyte maturation rates were very similar, making W2′82 an excellent control for evaluation of the developmental stages and determination of the type of defect.

Light Microscopy Studies of Gametocyte Development. *P. falciparum* gametocytes mature over a 1–2-wk period through a progression that can be followed by light microscopy. Five morphological stages (I–V) are described for Giemsa-stained gametocytes (4, 10, 11). In examining gametocytes from the Dd2 and W2′82 clones, we found marked differences in the production of mature Dd2 stages relative to those of W2′82. Whereas the W2′82 clone was a particularly good producer, regularly generating high numbers of synchronized and healthy-appearing gametocytes, Dd2 cultures were characterized by frequent abnormal and degraded gametocyte forms. Forms having abnormal shape and cytoplasmic color, condensed nuclei and inappropriate pigment organization, first apparent at stage III, were especially prominent at stages IV and V, coexisting with morphologically normal Dd2 gametocytes (Fig. 2). Particularly noticeable among these abnormal gametocytes were rectangular and striated forms with an acentric nucleus displaced from pigmented regions. In three independent experiments, these morphological differences in stage III–V Dd2 and W2′82 gametocytes were easily distinguished by different investigators from whom the identity of the cultures was concealed.

Defective Production of Male Gametocytes by the Dd2 Clone. The observation that the Dd2 clone always acted as the maternal parent in an outcrossing experiment (37) and

Table I. Counts of Oocysts in A. freeborni Mosquitoes 1 wk after Feeding with W2′82, 3D7, and Dd2 Gametocytes

Clone	Oocyst counts in individual mosquito midguts	Geometric mean
First day feeding		
W2′82 (exp 1)	21, 24, 55, 33, 18, 71, 33, 6, 11, 12	22.6
W2′82 (exp 2)	13, 5, 5, 5, 1, 5, 5, 0, 8, 33	5.1
Dd2 (exp 1)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0.03
Dd2 (exp 2)	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0.00
3D7	17, 22, 31, 27, 15, 26, 24, 1, 4, 16, 23, 11, 23, 32, 11, 5, 6, 23, 27, 10, 18	14.6
Second day feeding		
W2′82 (exp 1)	26, 6, 11, 1, 63, 12, 17, 8, 22, 2	10.6
W2′82 (exp 2)	18, 1, 2, 30, 1, 12, 19, 0, 5, 5	5.1
Dd2 (exp 1)	0, 0, 0, 0, 0, 0, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 1, 0, 0, 0, 0,	0.05
Dd2 (exp 2)	0, 0, 0, 0, 0, 0, 0, 2, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0,	0.03
3D7	12, 34, 16, 37, 26, 5, 46, 20, 29, 54, 11, 39, 15, 141, 8, 22, 32, 31, 28, 9	23.4

Mosquitoes were fed with mature gametocytes of the indicated clone on two consecutive days corresponding to the peak of exflagellation (17th and 18th days of culture for W2′82 [exp 1], W2′82 [exp 2], and Dd2 [exp 1], and 16th and 17th days of culture for Dd2 [exp 2] and 3D7). 7 d later, mosquitoes that had engorged were dissected and the oocysts in their midgut counted. Labels (*exp 1*) and (*exp 2*) correspond to two independent experiments conducted in parallel by different investigators with W2′82 and Dd2 gametocytes.

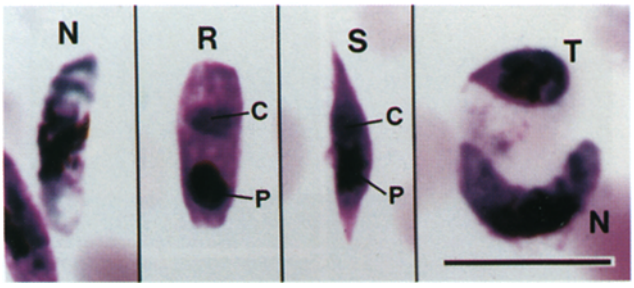


Figure 2. Abnormal gametocyte forms of Dd2 clone. Representative rectangular (*R*), spindle-shaped (*S*), and tear-drop (*T*) forms are shown alongside normal mature female gametocytes (*N*). Note the striated cytoplasm of the rectangular form and the clumped pigment (*P*) separated from the chromatin area (*C*). The clumped pigment, as well as the condensed cytoplasm of the spindle-shaped and the tear-drop parasites, distinguish them from normal younger stages. Bar, 10 μm.

that this was linked to a poor exflagellation and mosquito-infectivity phenotype (38) suggested to us that the production of mature male gametocytes could be defective. We therefore compared male and female gametocytes of the Dd2 and W2′82 clones. Fig. 3 *A* shows examples of normal male and female gametocytes distinguished by Giemsa staining. Stage V male and female gametocytes exhibit distinctive features: females have a blue cytoplasm and a condensed red nuclear area containing clumped pigment, whereas males have a pink cytoplasm and a wider nuclear area with a characteristic orange tinge. Since a major difference between mature male and female gametocytes lies in the abundance of cytoplasmic RNA, we also used propidium iodide to compare male and female gametocytes. Fig. 3 *B* shows that the uptake of this nucleic acid dye is much greater in blue gametocytes (female) than in pink gametocytes (male), confirming the results of Giemsa staining at the level of individual parasites. Separate experiments confirmed that the propidium iodide stain was removed by RNase treatment (not shown).

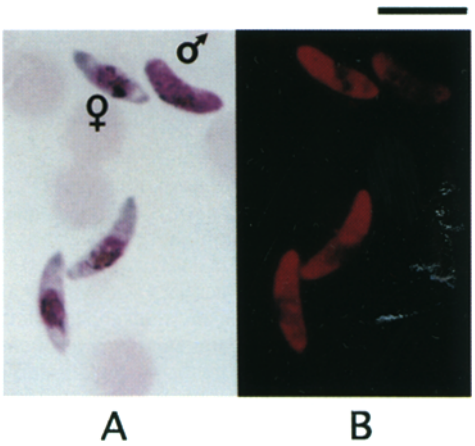


Figure 3. Normal mature male and female gametocytes detected by (*A*) Giemsa and (*B*) propidium iodide staining. Images of one male and three female gametocytes from clone W2′82 are shown. Bar, 10 μm.

We counted male and female gametocytes in Giemsa-stained preparations to compare the production of mature sexual stages by the Dd2 and W2′82 clones. These counts included only morphologically normal stage V gametocytes, as determined by the color and texture of the cytoplasm, the aspect and organization of the nuclear area, and the overall outline of the cell (4). Results of these experiments showed that the relative production of Dd2 male gametocytes was strikingly reduced compared to the normally infective W2′82 clone (Fig. 4). In three independent experiments, W2′82 male gametocytes were found to account for 7–12% of the total number of mature gametocytes, a percentage typical of a robust gametocyte-producing line, e.g., 3D7. Dd2 yields, by contrast, were reduced by 70–90% relative to those of W2′82. These ratios were consistent over days 14–19 of culture, indicating that the low male gametocyte counts were not the consequence of different maturation rates in culture (Fig. 4).

Detection of Male Gametocytes by Antibodies Against α-Tubulin II

The tubulin family of *P. falciparum* includes two isomers of α-tubulin that are differentially transcribed in sexual and asexual stage parasites (7, 14, 29). Immunostaining has shown that expression of α-tubulin II predominates in male gametocytes (stages III–V) and male gametes (29). A rabbit antiserum against the α-tubulin II COOH-terminal sequence (NH_2-CEGEDEGYEY-COOH) also has been shown to detect male gametocytes and gametes but not females or asexual stages (Fried M., and D.C. Kaslow, manuscript in preparation). We examined reactivity of anti–α-tubulin II COOH-terminal antibodies to Dd2 and W2′82 gametocytes, which were counterstained with antibodies against the general gametocyte surface protein Pfs230 (31). The appearance and relative numbers of the W2′82 gametocytes (Fig. 5, *A*, *B*, and *E*) were found to be

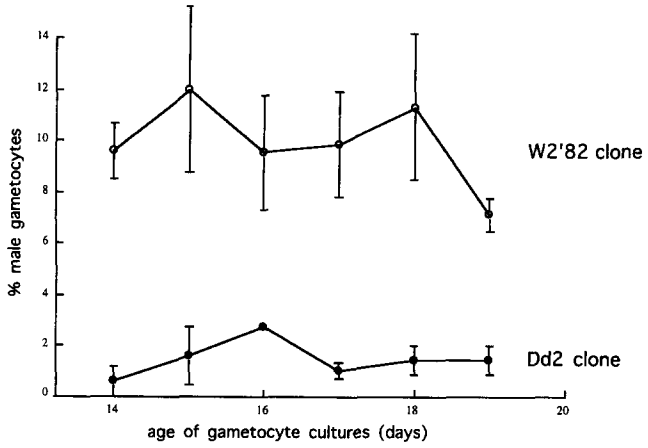


Figure 4. Reduced proportion of Dd2 male gametocytes detected by Giemsa staining. The proportion of male gametocytes is expressed as a percentage of the total number of gametocytes. Only normal mature gametocytes were counted. Data points and ranges represented by the bars indicate the arithmetic means from three independent experiments ± standard errors. At each day and for each experiment, the difference between the W2′82 and Dd2 counts was significant with a p value < 0.005 (χ^2 test, one degree of freedom).

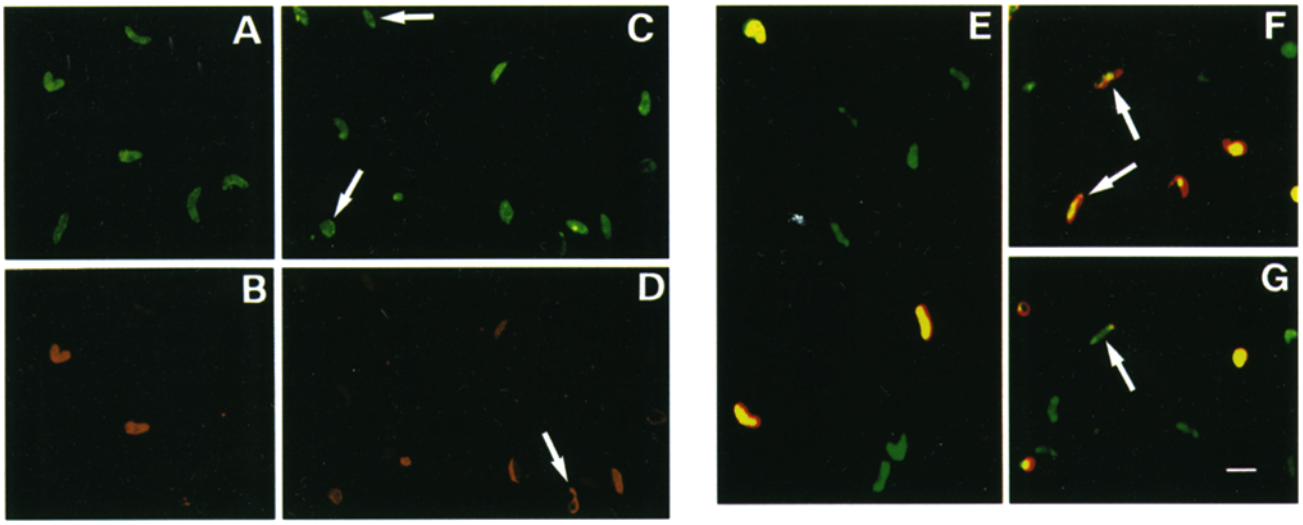


Figure 5. Immunofluorescence images of W2′82 (*A*, *B* and *E*) and Dd2 (*C*, *D*, *F*, and *G*) gametocytes detected by anti-Pfs230 and anti-α tubulin II male-specific antibodies. (*A* and *C*) Anti-Pfs230 signals. (*B* and *D*) Anti–α-tubulin II signals. In (*C* and *D*), the arrows mark stumpy, rounded, and fragmented forms from the Dd2 line. In *E*, *F* and *G*, two fluorescence images of the same field have been combined, resulting in distributions of yellow color that indicate the overlap of the green and red signals. Examples of variable expression of α-tubulin II and Pfs230 in the Dd2 parasites are marked by arrows. Bar, 10 μm.

indistinguishable from those of 3D7 (data not shown). In both the W2′82 and 3D7 preparations, the number of gametocytes detected by anti–α-tubulin II (10–12% from counts of several hundred gametocytes) represented a normal percentage for a male population, and male and female forms revealed by anti-Pfs230 staining had normal appearances.

By contrast, indirect immunofluorescence assays (IFA) of Dd2 gametocytes identified abnormalities consistent with those detected by Giemsa staining and by electron microscopy (following section). Anti-Pfs230 detected numerous stumpy, rounded, or fragmented forms of irregular size together with Dd2 gametocytes of normal appearance (Fig. 5, *C*, *F*, and *G*). Anti–α-tubulin II staining did not show the crisp distinction found between males and females of the W2′82 clone but instead showed a range of light, intermediate, and strong staining (Fig. 5, *D*, *F*, and *G*). Most of the irregular and fragmented forms displayed some staining while, among gametocytes of regular shape, normal positive signals were much less frequent in Dd2 than in W2′82 preparations.

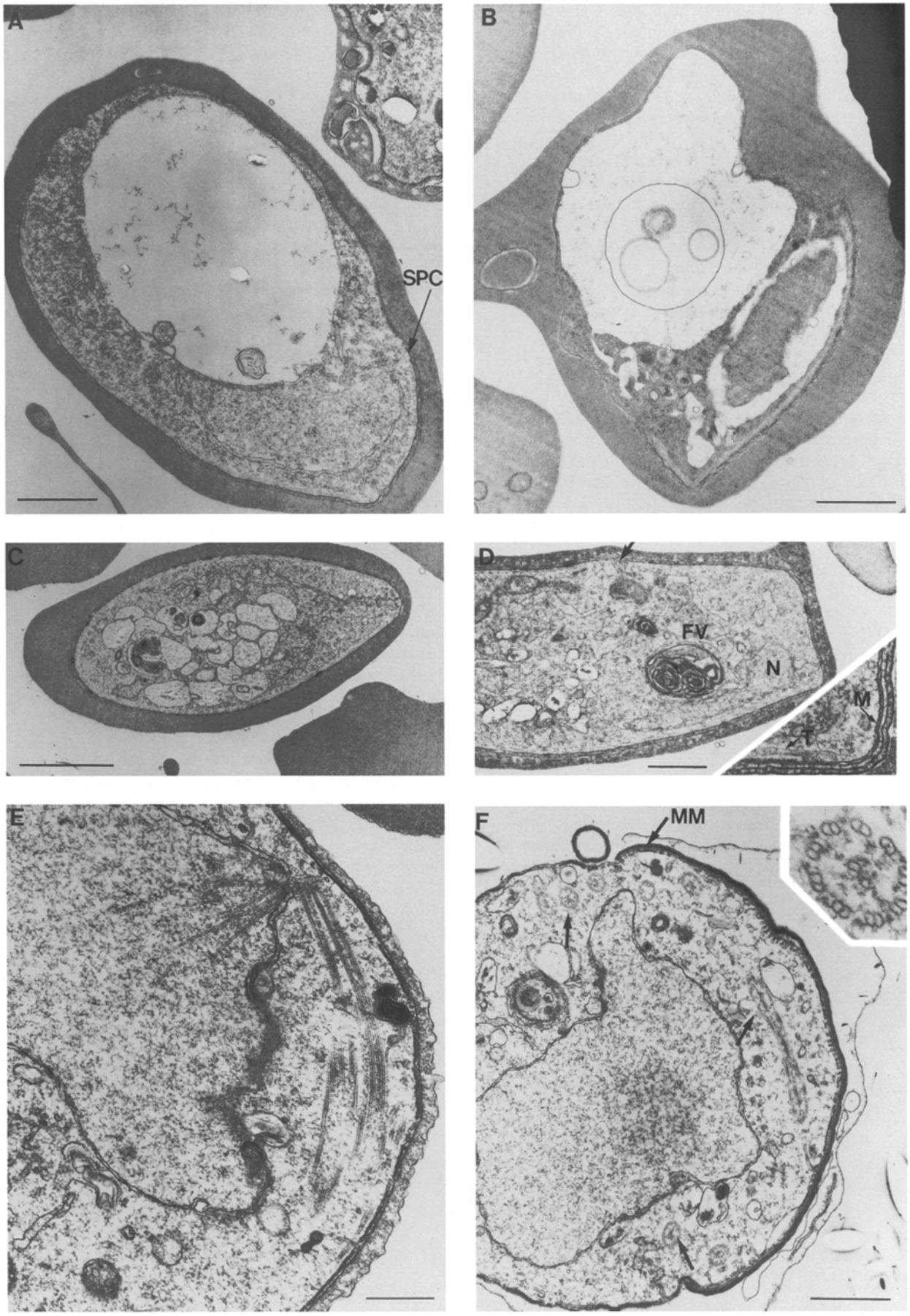
In the Dd2 mutant as well as in the two control clones, no positive signals were detected with control antibodies against the maltose binding protein or KLH alone (data not shown).

Ultrastructural Studies of Gametocyte Development

To further examine the abnormalities in the Dd2 gametocytes, we used electron microscopy to study samples fixed 10, 12, and 14 d after induction. Stage II, III, and IV gametocytes were observed in the preparations.

Compared to the electron micrographs of W2′82 and 3D7 gametocytes, images of Dd2 gametocytes showed irregular sizes and shapes, consistent with the abnormalities detected by IFA and light microscopy. Prominent in the ultrastructure of the Dd2 gametocytes were vacuolated regions of unusually large size, in some cases almost completely filling the parasite sections (Fig. 6, *A* and *B*). The presence of subpellicular double membrane and microtubules confirmed that images were of sexual stages (32, 34) and not of Dd2 asexual stages, which had a completely

Figure 6. Electron micrographs of gametocytes from the W2′82 and the Dd2 lines. (*A* and *B*) Images from sections of abnormal Dd2 parasites, each almost completely filled with a large vacuole. In *A* the subpellicular complex (*SPC*) lines about half of the parasite surface. In *B* the remaining cytoplasm is denser than normal and contains few ribosomes. Note the intravacuolar membranes that may arise by blebs from the internal side of the vacuole, as seen at the left of the image. (*C*) Transverse section of a stage III–IV Dd2 gametocyte showing multiple vesicles. The appearance of some vesicles suggests fusion events that may give rise to larger vacuoles. (*D*) Image of a "rectangular shaped" Dd2 gametocyte with angled corners. A cytostome (*arrow*) is visible. The elongated shape and the subpellicular complex (membrane + microtubules) underlying the plasma membrane indicate stage III–IV maturity. (*Inset*) 3.5× enlargement of an angled corner, showing the normal appearance of the subpellicular membrane and microtubules. *FV*, food vacuole; *N*, nucleus; *T*, subpellicular microtubules; *M*, subpellicular membrane. (*E*) Activated male W2′82 gametocyte 10 min after induction. An intranuclear spindle and cytoplasmic axonemal microtubules are visible. Connection of the centriolar plaque and the kinetosome through a nuclear pore is as described for *P. yoelii* (33). (*F*) Activated male Dd2 gametocyte 10 min after induction. Note the transverse sections and longitudinal section of axonemes (*arrows*), which exhibit the classical 9 + 2 organization (*Inset* enlargement 6.45). A multilamellate system of six membranes (*MM*) lies close to the rounded, extraglobular parasite. Such a structure has been described in the cytoplasm of erythrocytes infected by mature gametocytes and is thought to be derived from the vacuolar membrane (28). Bars: (*A*, *B*, *D*, and *F*) 1 μm; (*C*) 2 μm; (*E*) 0.5 μm.


A
SPC
B
C
D
FV
N
M
T
E
F
MM

normal ultrastructural appearance. Whereas some of the vacuolated gametocytes were surrounded by the inner double membrane and the underlying microtubules, others exhibited only a short double membrane structure, indicating abnormalities in development as early as in stage II.

Some micrographs (Fig. 6 *C*) showed images of multiple small vacuoles in possible states of fusion, which suggests they may participate in the development of giant vacuoles. Longitudinal sections of Dd2 gametocytes also revealed rectangular shapes reminiscent of the rectangular gametocytes noted by light microscopy (Fig. 6 *D*). In W2'82 and 3D7 electron-microscopy sections, images of rectangular gametocytes were much less frequently observed. At the resolution of the electron image, we did not detect obvious abnormalities accounting for the striated cytoplasm or the angular features of the rectangular forms. In particular, no ultrastructural abnormalities were evident in the pellicular–microtubule complex (Fig. 6 *D*, *Inset*), which is thought to be responsible for the general shape of the gametocytes (32). The rectangular forms displayed features consistent with the male pathway, including relatively low numbers of organelles, even when the development of the pellicular–microtubule complex indicated stages late III or IV and large, convoluted nuclei.

Samples of stage V Dd2, W2'82, and 3D7 gametocyte cultures were also examined by electron microscopy at different times (1, 3, 5, 10, and 15 min) after induction of exflagellation. In the W2'82 and 3D7 control preparations, parasites in various stages of exflagellation were readily identified (Fig. 6 *E*), including emerging male gametes. By contrast, very few early stages of exflagellating parasites were found in the Dd2 preparations: some microtubules and axoneme structures were detected in rounded gametocytes free of the red blood cell, but the subsequent events of kinetosome–axoneme associations and nuclear segregation were not observed (Fig. 6 *F*).

Although there are ultrastructural features that can distinguish normal male and female gametocytes (34), the disorganized internal architecture of the abnormal forms generally precluded accurate evaluations of sex ratios by electron microscopy.

Inheritance of the Dd2 Gametocyte Development Defect in a Genetic Cross

In previous studies, we showed that poor infectivity of Dd2 parasites to mosquitoes and reduced exflagellation were linked to a chromosome12 RFLP marker (pL5.7–26) in the *P. falciparum* cross of clones Dd2 and HB3 (38). The finding in the present work that these defects traced to developmental abnormalities of the Dd2 gametocytes led us to examine inheritance patterns of the gametocyte production phenotype in the HB3 × Dd2 cross. Table II lists the percentages of male gametocytes, mosquito infectivities, and pL5.7–26 marker types for nine independent-recombinant progeny. The male gametocyte production phenotypes of HB3 (normal) and Dd2 (defective) were clearly distinguishable and were linked to mosquito infectivity and pL5.7–26 inheritance. As determined by Giemsa staining, the percentage of male gametocytes produced by normal progeny was $\geq 10\%$, whereas the percentage from progeny with the defective Dd2 character was $\leq 3.5\%$, with a significant difference between the two groups ($P < 0.005$). Frequent abnormal forms were usually associated with the defect. The data thus indicate that abnormalities in male gametocyte production and mosquito infectivity are linked to the same chromosome segment and that a single gene or a closely associated group of genes accounts for the alterations in phenotype.

Discussion

The results of this work show that the male gamete defect of the Dd2 clone occurred during long term cultivation in vitro and that it is linked to a developmental mutation affecting mature male gametocyte production. Availability of the W2'82 and Dd2 clones as an isogenic pair differing in gametocyte development provides an important basis for comparative phenotypic and molecular studies to characterize the defect. This resource coupled with linkage analysis of the HB3 × Dd2 cross may open up routes of investigation into sexual stage differentiation that previously have not been available.

The Dd2 defect is characterized by a reduced production of mature male gametocytes relative to the normal yield from W2'82 and an another unrelated control clone, 3D7. Repeated Giemsa counts in separate experiments, supported by data from electron microscopy and IFA with male-specific anti-α-tubulin II antibodies, show that the proportion of mature Dd2 male gametocytes is reduced to <10–30% of the normal value. The point at which the sex ratio imbalance arises is not determined, although evidence from the electron micrographs suggests the presence of abnormalities as early as stage II. The functional defect itself is manifested specifically by males: development of Dd2 asexual stages was found to be entirely normal, and female gametocytes were fertilized as expected in the HB3 × Dd2 cross (37–39).

Table II. Genetic Linkage of Male Gametocytogenesis to Oocyst Production and to a Segment of Chromosome 12

Progeny clone	B1-SD	QC-13	3B-B1	SC-05	GC-03	3B-D5	SC-01	QC-34	GC-06	QC-23	TC-05
pL5.7-26 type	H	H	D	H	D	D	D	H	D	H	D
Mean No. oocysts per mosquito	4.3	12.0	0	6.1	0.4	0	0.5	5.7	0.1	3.2	0.1
Percentage mature gametocytes*	11.7 ± 2.0	10.7 ± 1.2	—	12.7	0.3 ± 0.3	—	3.0	31.5 ± 2.9	3.5 ± 0.1	13.9 ± 1.3	1.0

HB3 or Dd2 types of the chromosome 12 polymorphic marker pL5.7–26 and the numbers of oocysts per mosquito are from reference (38). Sex ratios were determined by review of slides of gametocyte preparations from that study.

*Percentages are from averages of counts obtained from gametocyte cultures over several days. Percentages are given as arithmetic means ± standard deviations for those progeny for which more than one gametocyte culture was examined. There is a statistically significant difference in the male gametocyte percentages ($P < 0.005$) from HB3-type and the Dd2-type progeny, as defined by their mosquito infectivity and pL5.7–26 polymorphisms.

The defect reported here is the first known mutation affecting the sex ratio of mature gametocytes in *Plasmodium*. Molecular mechanisms of homothallism have been studied mainly in budding and fission yeasts (9, 12), where mating-type determination has been linked to the switching of cassettes containing master regulatory genes. There is no evidence whether or not such a strategy is used by *Plasmodium* spp., which produce, in unequal number, two morphologically and functionally distinct sexual forms that must undergo a prolonged maturation and differentiation period before they are competent for fertilization. In plants, certain forms of male sterility have been associated with mitochondrial mutations resulting in the generation of novel open reading frames (5, 22). However, in the *P. falciparum* clone described here, the genetic evidence clearly points to a linkage group in chromosome 12 of the nuclear genome and not to a cytoplasmic marker.

In addition to the reduced production of mature male gametocytes observed in this study, frequent abnormal gametocytes of irregular size were characteristic of Dd2 cultures. These abnormal forms were readily observed by both light and electron microscopy and were not a feature of the W2'82 cultures. Irregular and varied IFA signals were obtained from the abnormal Dd2 forms incubated with male-specific anti-α-tubulin II antibody, in contrast to the normal signals that distinguished normal mature male and female gametocytes of the W2'82 and 3D7 lines. The generation of abnormal forms by the Dd2 line is likely to be part of the process by which the production of mature males is selectively impaired, although involvement of female gametocytes to some extent cannot be ruled out. The most straightforward hypothesis is that a genetic change that involves a single gene or a closely linked group of genes is responsible for both defective male gametocyte production and the abnormal forms. Preliminary observations indicate the two phenotypic traits tend to track together in the HB3 × Dd2 progeny, although for some defective clones we have observed less prominent production of abnormal forms than for Dd2.

Is the diminished representation of mature male gametocytes the only factor accounting for the massive defect in exflagellation and mosquito infectivity? Do additional male functional abnormalities, not detectable by morphological examination of gametocytes, further interfere with mosquito-specific steps? Quantitative correlations between gametocytogenesis, exflagellation, and oocyst production cannot be easily obtained because of the multiplicity of the factors involved (16, 23, 27). Although early stages of the exflagellation process were apparent by electron microscopy in a limited number of Dd2 parasites, it may be significant that these showed only the first steps of rounding up and axonemal organization, while in the control clones various exflagellation stages, up to the emergence of microgametes, were observed.

The genetic linkage to chromosome 12 of the impairments of gametocytogenesis, exflagellation, and mosquito infectivity may reflect the presence of a gene or gene cluster involved in sexual development. It is not known whether *P. falciparum* genome has discrete chromosome regions that govern male and female differentiation. Of note from studies of *P. berghei* is that three genes expressed during early sexual development have been mapped to chromosome 5 and deletions from part of this chromosome have been associated with a loss of gametocyte production (17, 18).

The defect characterized in this study should be identifiable by positional cloning strategies directed to chromosome 12 and DNA comparisons of the Dd2 and W2'82 clones. Advances in the use of microsatellite methods for linkage mapping (35) and in the cloning of high numbers of progeny from parasite crosses (20) can now be used to localize genetic determinants within 50 kb. Detailed analysis of a chromosomal region of this size should be achievable by sequencing and transfection methods (44, 45) and should contribute to a better understanding of the molecular events that direct sexual differentiation in malaria parasites.

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