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SEASONAL FLUCTUATION OF ANTIBODY LEVELS TO *PLASMODIUM FALCIPARUM* PARASITIZED RED BLOOD CELL-ASSOCIATED ANTIGENS IN TWO SENEGALESE VILLAGES WITH DIFFERENT TRANSMISSION CONDITIONS

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Abstract. The recombinant R23, PfEB200, and GST-5 antigens derive from conserved antigens associated with the *Plasmodium falciparum*-infected erythrocyte membrane. They were identified as targets of protective antibodies in the *Saimiri sciureus* model. We have assessed here the humoral response to these antigens in humans. Cross-sectional surveys were conducted in two Senegalese villages with different levels of endemicity. The prevalence of specific IgG and IgM was similar and influenced by age in both localities. The anti-R23 antibodies decreased after the rainy season, particularly in the children less than ten years old. The anti-PfEB200 response did not show significant seasonal variation. The anti-GST-5 response increased in both the less-than 10-year-old and the greater-than 10-year-old groups after the rainy season in Dielmo, but only in the Ndiop villagers who were more than 10-years-old. Thus, antigen-specific seasonal variations of antibody levels were influenced differently by age in both villages. The isotype distribution was antigen-specific and differed for both seasons.

INTRODUCTION

Several lines of evidence indicate that antibodies reacting with the surface of the infected red blood cells contribute to the protection against *Plasmodium falciparum*.[1,2] In *Saimiri sciureus*, a New World monkey used as an experimental model of infection by asexual *P. falciparum* blood stages, protection positively correlates with the presence of antibodies promoting opsonization of the infected red blood cell.[3,4] Evidence has been obtained that such an effector mechanism also exists in immune humans.[5] Interestingly, sera from individuals living in *P. falciparum*-endemic areas opsonize infected *Saimiri* red blood cells,[6] suggesting that the parasite antigens exposed on the surface of the monkey red blood cells are naturally immunogenic in man.

A number of recombinant proteins have been screened for their capacity to compete for antibody (Ab) with the infected red blood cell (IRBC) in an *in vitro* immune phagocytosis assay.[7] This identified the R23 and PfEB200 antigens as targets of opsonizing antibodies. R23 derives from the central domain of the R45 antigen: it consists of 11 copies of a 6-amino-acid motif.[8] The R45 protein contains an N-terminal signal sequence and a putative transmembrane domain located close to the C-terminus (Genbank ALO 10255). The central domain is exposed on the surface of infected red blood cells, as indicated by a positive surface fluorescence observed in FACScan analysis using specific *Saimiri* sera.[9] PfEB200 derives from Pf332, a conserved large protein associated with the IRBC membrane, containing a large number of repeats with characteristic Glu-Glu dimers. The PfEB200 antigen contains 13 such repeats. This antigen is accessible on the infected red blood cell surface in late shizonts.[10] Both antigens are conserved in all strains examined so far. Immunization of *Saimiri* monkeys using the PfEB200 and R23 recombinant glutathione-S -transferase (GST) proteins elicited Abs mediating immune phagocytosis of IRBC *in vitro* and protected monkeys from a lethal *P. falciparum* infection i.e., the Uganda Palo Alto strain adapted to splenectomized monkey (FUP/SP).[9,11]

PfEMP3 is a conserved IRBC membrane antigen, recently identified as a potential target of protective Abs in the *Saimiri* model. Indeed, its expression level is modulated during antigenic variation, with some variants expressing high levels while others express reduced amounts of the protein.[12] Consistent with this, clone 5 (GST-5), a recombinant clone expressing a fragment derived from the PfEMP3 C-terminal domain, was identified as a target of a variant-specific immune response.[13] Thus, the three antigens are targets of protective antibodies to *P. falciparum* blood stages and as such represent interesting vaccine candidates.

In order to investigate whether *P. falciparum* infections also elicit an immune response to these antigens in humans, we examined the humoral response to the R23, PfEB200, and GST-5 antigens in individuals living in two Senegalese neighbouring villages with different levels of malaria endemicity. In Dielmo, transmission is intense and perennial,[14,15] whereas in Ndiop transmission is moderate and interrupted during the 8–11 months of the dry season.[16,17] Comparative studies in Dielmo and Ndiop have outlined major differences in the age incidence of clinical malaria [14,16] and the molecular characteristics of the infections.[18,19] Thus, parallel investigations in villagers from both localities permit an appreciation of the respective influence of age and cumulative exposure to parasites on the immune responses.[20] We have analysed here the prevalence and the dynamics of specific antibody responses to R23, PfEB200, and GST-5 antigens in 88 matched individuals of various ages recruited in two cross-sectional surveys conducted during the dry season and shortly after the rainy season.

MATERIALS AND METHODS

Recombinant proteins. The recombinant antigens were prepared as soluble proteins fused to GST in the pGEXA vector. They were purified by affinity chromatography on glutathione agarose beads (Sigma Chemicals, St Louis, MO) as described.[21]

R23 contains 11 copies of a 6-amino-acid motif derived

from the central repeat domain of antigen R45 for which the consensus sequence is HKSDS N/S/H.[8] PfEB200 contains a 135-amino-acid sequence derived from antigen Pf332, consisting of 13 degenerate copies of a 11-amino-acid repeat (consensus sequence GSVTEEIVE/QEE).[10] GST-5 is the recombinant product expressing the 1450 bp *EcoR*I fragment recovered from clone 5 isolated from a FUP/SP Palo Alto genomic expression library. It includes the C-proximal region of PfEMP3, namely 5 copies of a 19-amino-acid repeat, 3 copies of a 15-amino-acid repeat followed by 24 copies of a 13-amino-acid repeat.[13] The control protein was the carrier GST. All proteins were aliquoted and stored at −20°C before use.

Study sites and subjects. Plasma was collected into capillary tubes from individuals living permanently in Dielmo and Ndiop. The enrolment of the villagers in a longitudinal study was approved by the National/Ministry of Health ethical committee; informed consent was obtained from all human adult participants and from parents or legal guardians of minors. Informed consent of the villagers was renewed every year and anyone was free to leave the study at any time.[14] Eighty-eight villagers 0.4–71 years old were selected in each village. They were matched for age, gender, and hemoglobin type (AA/AS). Initial sampling from the 88 donors in each village (0–10 yr: n = 31; >10 yr: n = 57) was done in March 1996. Most individuals were also bled in December 1996, i.e., 85 villagers from Dielmo (0–10 yr: n = 28; >10 yr: n = 57), and 76 villagers from Ndiop (0–10 yr: n = 28; >10 yr: n = 48).

During 1996, the entomological inoculation rate (EIR) recorded in Ndiop was 7 *P. falciparum* infective bites per individual per year. The March survey conducted during the dry season corresponded to a period with no recorded transmission for the previous 5 months in Ndiop. The December survey occurred 2 months after the transmission season, which lasted a single month (September 1996). The annual entomological inoculation rate (EIR) recorded in 1996 in Dielmo was 305 *P. falciparum* infective bites per individual. Transmission intensity fluctuated over the year.[15] The EIR was 17, 18, and 15 *P. falciparum* infective bites per individual per month in the 3 months preceding the March 1996 survey (December 1995, January 1996, and February 1996, respectively). The December 1996 survey was conducted after the rainy season. In the preceding 3 months, the recorded EIR was 54 (September 1996), 39 (October 1996), and 18 (November 1996).

In both villages, all clinical episodes were recorded on a daily basis for the study period.[14,16] Clinical events with an axillary temperature >38°C (and possibly additional symptoms) and a *P. falciparum* parasite density above a certain threshold were considered malaria attacks. The pyrogenic parasite density threshold differed in both villages. For Dielmo, we used the age-dependent threshold previously established.[22] For Ndiop, the threshold was determined as 2,800 trophozoites/μl (Spiegel A, and others, unpublished data).

Enzyme-linked immunosorbent assay (ELISA). Specific antibody responses were measured by indirect ELISA.[20,23] Unless otherwise stated, all reagents were purchased from Sigma Chemicals. Briefly, 96-well Immulon-4 plates (Dynatech, Springfield, VA) were coated overnight at 4°C with 100 μl protein (1 μg/mL) diluted in PBS 1x (the control GST was coated at 0.7 μg/mL). After washing and blocking with PBS/5% bovine serum albumin (BSA) for 1 hour at 37°C, plasma samples diluted 1:200 in PBS/1% BSA / 0.5% Tween 20 were added in duplicate for 2 hours at 37°C. After washing, peroxidase-conjugated polyclonal goat anti-human IgG (γ chain specific) or IgM (μ chain specific) was added for 2 hours at 37°C (1/10,000; Cappel: Organon Teknika, Turnhout, Belgium). Following incubation and five washings, 100 μl citrate buffer (pH 4) containing 0.16 mg/mL ortho-toluidine and 10% H_2O_2 was distributed in each well. The reaction was stopped using 4N H_2SO_4 and the optical density (OD) was read using a Titertek Multiscan plate reader (Flow Laboratories, Ayshire, Scotland, UK). For the determination of IgG subclasses, mouse monoclonal Abs were used as reported[20] and revealed using peroxidase-labelled goat anti-mouse IgG. For R23 and PfEB200, subclass distribution was investigated for all Dielmo and Ndiop samples with an IgG OD value > 0.5. For GST-5, a representative fraction of sera was assayed from both villages. In every plate, a negative control (a reference European serum pool) and a positive control (a pool of sera from clinically immune adults living in Dielmo and Ndiop) were included. Inter-assay variations for positive controls did not exceed 15%.

Positivity threshold determination. Since the recombinant proteins were C-terminal fusions to GST, the reactivity to the carrier moiety was subtracted from OD values obtained with each recombinant protein. The mean ± standard deviation (SD) OD level to GST in 170 samples from Europeans without history of malaria was 0.1 ± 0.04. For the villagers, the mean level of IgG to GST was low during both the dry and the rainy season (0.032 ± 0.039 and 0.057 ± 0.07 in Dielmo, and 0.043 ± 0.1 and 0.049 ± 0.067 in Ndiop, in March and December, respectively). In 22 European sera, the mean OD value for IgG to the recombinant Ags was 0.03 ± 0.03. In view of these data, a net 0.1 OD value difference (recombinant minus GST signal) was taken as the "positive" threshold of Ab responses to the parasite moiety of the recombinant GST fusion proteins. The anti-GST values were slightly more elevated for the IgM (0.26 ± 0.3 in the Dielmo series and 0.20 ± 0.23 in the Ndiop series). In European sera it was 0.04 ± 0.028.

Statistical analysis. Statistical comparisons between Ab levels expressed as mean individual OD were done by means of the Wilcoxon signed rank test and the Spearman rank correlation for non-normally distributed paired data. *P* values < 0.05 were considered significant. The relationship between occurrence of clinical malaria episodes, incidence rate, and Ab responses was tested using the Chi square test (Epi-Info 6 software, Centers for Disease Control and Prevention, Atlanta, GA); *P*-values were considered significant after calculation using the Bonferoni correction, namely < 0.05/n where n is the number of comparisons.

RESULTS

Seroprevalence in Dielmo and Ndiop. The prevalence of IgG and IgM to R23, PfEB200 and GST-5 is shown in Figure 1. For each antigen, the IgG or IgM prevalence and the seasonal variations were remarkably similar in both villages. The only significant inter-village difference was the propor-

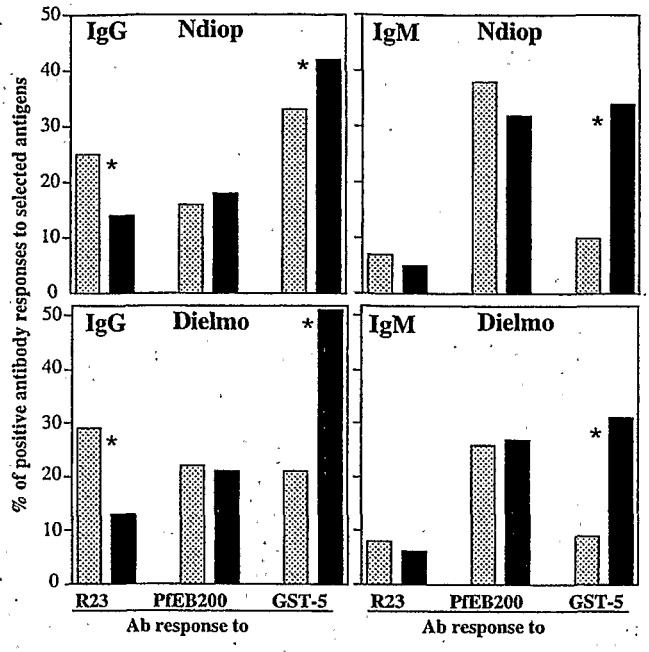

FIGURE 1. Incidence of IgG and IgM antibody responses to the R23, PfEB200, and GST-5 recombinant antigens by season in the villages of Ndiop and Dielmo. Percentage of individuals with a positive antibody response in March (grey histograms) and December (black histograms) is plotted for each village. The specific antibodies were assayed for the same matched individuals for the two surveys. An asterisk * next to a histogram indicates seasonal response levels that are significantly different ($P < 0.05$). Ab = antibody

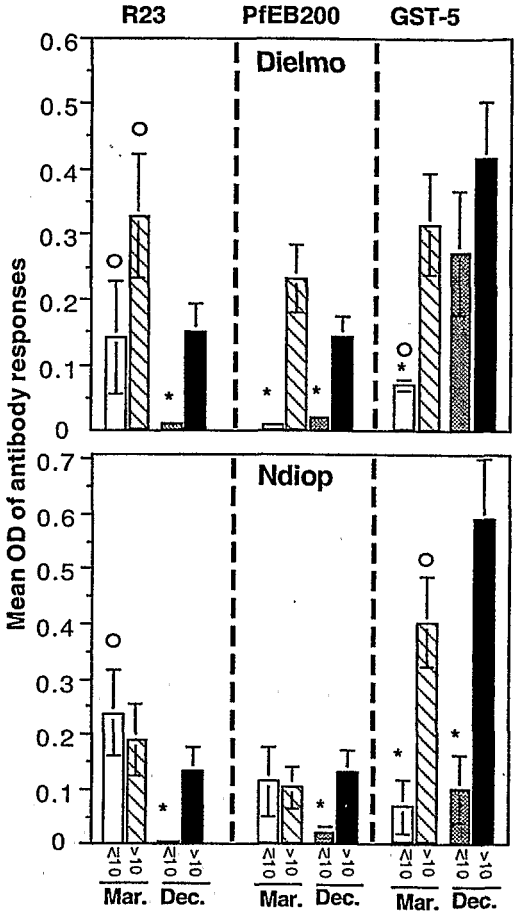

FIGURE 2. IgG antibody responses to the R23, PfEB200, and GST-5 recombinant antigens by age group in the villages of Ndiop and Dielmo. The mean optical density (OD) of IgG responses from all individual plasmas collected in March 1996 (Mar.) and December 1996 (Dec.) for each age group are plotted. The age groups (≤ 10-yr and > 10-yr) is indicated under the histograms. An asterisk (*) above a histogram indicates a significant difference in the age distribution ($P < 0.01$). A round symbol (o) above a histogram indicates seasonal variation that is considered statistically significant for that particular age group in that village.

tion with anti-GST-5 IgG, which was higher in Ndiop than Dielmo during the March survey ($P = 0.023$).

There were seasonal fluctuations in seroprevalence and these were antigen-specific. The percentage of anti-R23 IgG Ab responses was reduced by 50% in December compared to March, decreasing from 25–29 % to 13–14% ($P < 0.01$). The anti-R23 IgM response was low and did not show any noticeable seasonal variation. The Ab response pattern to PfEB200 differed from the anti-R23 response. In both surveys and in both villages, the number of villagers with PfEB200-specific IgM was higher than those with PfEB200-specific IgG ($P < 0.01$). There was a limited impact of the season on both PfEB200-specific Ab classes. The response to GST-5 presented a third pattern, with a significant increase in prevalence of both specific IgG and IgM in December compared to March ($P < 0.01$). The mean OD values to GST-5 also increased in the second survey ($P < 0.01$).

Influence of age on IgG antibody responses to R23, PfEB200, and GST-5. 85 individuals in Dielmo and 76 in Ndiop who donated blood in March and December entered the matched comparison study. Two age groups were considered, ≤ 10 and > 10 years old, based on the mean age at which the incidence of clinical attacks is reduced in both villages.[16] The mean OD for specific IgG by age group in both surveys is indicated in Figure 2. The antigen-specific responses were influenced by age. The seasonal fluctuations were influenced differently by age in both villages.

The anti-R23 response did not differ in both age groups in March (Dielmo, $P = 0.19$; Ndiop, $P = 0.98$) but was significantly higher in the older age group in both villages in December ($P < 0.01$). In both villages, no anti-R23 response was observable in the children ≤ 10 years old in December. This decrease compared to March was statistically significant ($P < 0.01$). A similar decrease was observed in the older age group in Dielmo ($P < 0.01$).

In Dielmo, an anti-PfEB200 response was evident in the older age group only ($P < 0.01$, for each survey). A trend for a decreased anti-PfEB200 mean value in December compared to March was observed but did not reach statistical significance ($P = 0.055$). In Ndiop, a low response was observed in both age groups in March. It was restricted to the older age group in December. There was no statistically significant seasonal variation ($P = 0.7$).

In both villages, the anti-GST-5 response was much higher in the older age group in March ($P < 0.01$). The profiles and mean OD values were very similar in both localities. In December, however, the age distribution differed in Dielmo and Ndiop. A marked increase of the anti-GST-5 mean OD in December compared to March was observed in the ≤ 10-yr-old children from Dielmo ($P < 0.01$) but not Ndiop. The

TABLE 1
IgG subclass distribution of anti-R23, PfEB200, and GST-5 according to season

IgG subclass	Number of villagers* responding to antigen (%)					
	R23		PfEB200		GST-5	
	March n = 18	December n = 8	March n = 14	December n = 11	March n = 17	December n = 12
IgG1	7 (39)	4 (50)	3 (21)	2 (18)	1 (6)	1 (8)
IgG2	13 (72)	0 (0)	8 (57)	0 (0)	8 (47)	1 (8)
IgG3	11 (61)	4 (50)	11 (79)	6 (55)	17 (100)	12 (100)
IgG4	1 (6)	1 (13)	0 (0)	2 (18)	7 (41)	2 (17)

* Data from the villages of Ndiop and Dielmo are pooled.

mean OD value tended to increase in the older age group (Dielmo, $P = 0.06$; Ndiop, $P < 0.01$).

Low Ab titers to R23 and PfEB200 (1/2,000) were observed for the positive plasma samples (samples with detectable IgG signal was > 0.5 OD). Titers (and probably affinity) to GST-5 were higher. This was observed in both villages and for both surveys.

Anti-R23, -PfEB200, and -GST-5 IgG subclasses. The antigen-specific IgG subclass distribution was similar in Dielmo and Ndiop and was antigen-specific (Table 1). A high proportion of seropositive individuals had R23-specific IgG2 and IgG3 and to a lesser extent IgG1 in March. However, anti-R23 IgG2 was not detected in December. A similar picture was observed for the anti-PfEB200 IgG subclasses. All GST-5 sero reactive subjects had IgG3 in March and December. In addition, IgG2- and IgG4-specific antibodies were detected in March only. Thus, IgG3 was the predominant anti-GST-5 subclass in the dry season survey.

Relationship of Ab responses to incidence of clinical malaria and recent infections. In view of the high prevalence of short-lived IgG3 Abs to the antigens studied, we focused the prospective analysis on the 3 month period following blood sampling. This restricted the analysis to Dielmo, where transmission and clinical attacks occurred year-round. During the 3-month period following the March 1996 survey, 46 clinical episodes were recorded of which 43 occurred in the younger age group. During the 3-month period following the December 1996 serological survey, 28 clinical episodes were recorded. All were in the younger age group. No definitive conclusion can be drawn. This is because of the low or null prevalence of responders in the < 10-yr-old group in which clinical attacks are clustered and accountable of the low incidence of malaria episodes in the older age group, in which a substantial proportion of individuals have seroconverted (data not shown).

In Ndiop, a prospective analysis cannot be done with these data since clinical attacks were clustered in a period of transmission that was distant from the March serological survey. We investigated whether clinical malaria, i.e., exposure to infections with high parasitemia, was associated with the Ab responses assayed in December. No association was found (data not shown).

DISCUSSION

Two distinct endemic conditions have been explored. In Dielmo, a setting where there are permanent breeding sites, *P. falciparum* transmission occurs year-round.[15] In contrast, transmission is strictly seasonal in Ndiop.[17] During 1996 both intensity and duration of transmission differed markedly in these villages. Therefore, Ab responses and seasonal fluctuations should be interpreted in light of the transmission conditions prevailing in those villages.

Not all individuals had detectable Abs to the recombinant proteins tested. A higher seroprevalence was reported for RESA, another IRBC membrane-associated antigen and for merozoite antigens, such as merozoite surface protein-1 (MSP-1) or apical membrane antigen-1 (AMA-1).[24–27] Whether the lower prevalence observed here reflects restricted immunogenicity of the R23, PfEB200, and GST-5 antigenic determinants is unclear. It is possible that the positivity threshold used underestimated the seroprevalence. The ELISA technique used here may be less sensitive than capture assays[27] or assays using signal amplification.[28] In addition, we cannot exclude the possibility that the recombinant proteins do not optimally mimic the native parasite antigens. However, the fact that the R23 and PfEB200 proteins competed efficiently with IRBC in the *in vitro* opsonization assay[7] indicates that some critical native epitopes are expressed by the recombinant antigens.

The observation of a similar overall prevalence of responders to either antigen in both locations is remarkable. This is consistent with previous observations in these villages for Ab responses to MSP-1[19,20] and with the observation of similar responses to MSP-1 in urban and rural settings in The Gambia.[26] As predicted, the specific Ab response was influenced by age. The impact of age on the response to the individual antigens was different in Dielmo and Ndiop and differed for each antigen. For the three antigens tested, the response was influenced by age in Dielmo, with a higher response in the older group in both surveys. This is consistent with the notion that age reflects cumulated exposure to infection and probably reflects the necessity for a substantial cumulative exposure for Ab production to these antigens. In Ndiop, the picture was more complex, with marked inter-survey differences. An age-dependent increase in Ab response was observed for R23 and PfEB200 in the December survey and for GST-5 in both surveys. This may reflect age-related differences in the impact of variation of transmission intensity on the dynamics of Ab levels.

Ab isotype distribution was antigen-specific but similar in Dielmo and Ndiop. This contrasts the observed difference of anti-MSP-1[19] isotype distribution in these localities.[20] An antigen-specific IgG subclass distribution of anti-R23, -PfEB200, or -GST-5 IgG was noted in this study. This indicates that factors that contribute to IgG subclass distribution in response to complex antigenic mixtures such as parasites are antigen-specific. An interesting observation was the consistent reduction of IgG2 Abs after the rainy season. Whether this reflects preferential production of the short-lived IgG3 subclass upon recent increased exposure to parasites is unclear. The identification of the parameters that regulate Ab isotype balance to *P. falciparum* is important as cytophilic Abs are associated with protection against clinical malaria, which itself is associated with low parasite densities.[29–31] Specificity of subclass distribution differed in both seasons, with no specific IgG2 detected in December.

There were antigen-specific seasonal fluctuations of specific Abs. In Ndiop, the rainy season is characterized by

recent exposure to parasites after a prolonged period without inoculation. In Dielmo it was associated with a 2.2-fold increase in transmission intensity. The anti-GST-5 Abs increased after the rainy season. This can be interpreted as boosting by recent or current exposure to parasites in partially immune individuals, as observed for other *P. falciparum* antigens.[26,27,32] It is surprising that recent increased parasite exposure did not result in an increase in antibodies to R23 and PfEB200. This illustrates the difficulty of interpreting serologic data from a single time point. The levels of Ab present at a single time reflect the net balance of Ab production and consumption of Ab by binding to parasites. It is difficult to evaluate the respective contribution of these opposing processes in cross-sectional surveys. The reduction of anti-R23 levels is in line with our observations of decreased anti-R23 Ab after challenge infection of R23-immunized *Saimiri sciureus* monkeys.[9,11] A negative correlation of Ab levels with parasite exposure is consistent with a role for the anti-R23 Ab in parasite clearance in humans. Careful longitudinal studies should clarify the dynamics of Ab levels and their relationship with the dynamics of parasite infection.

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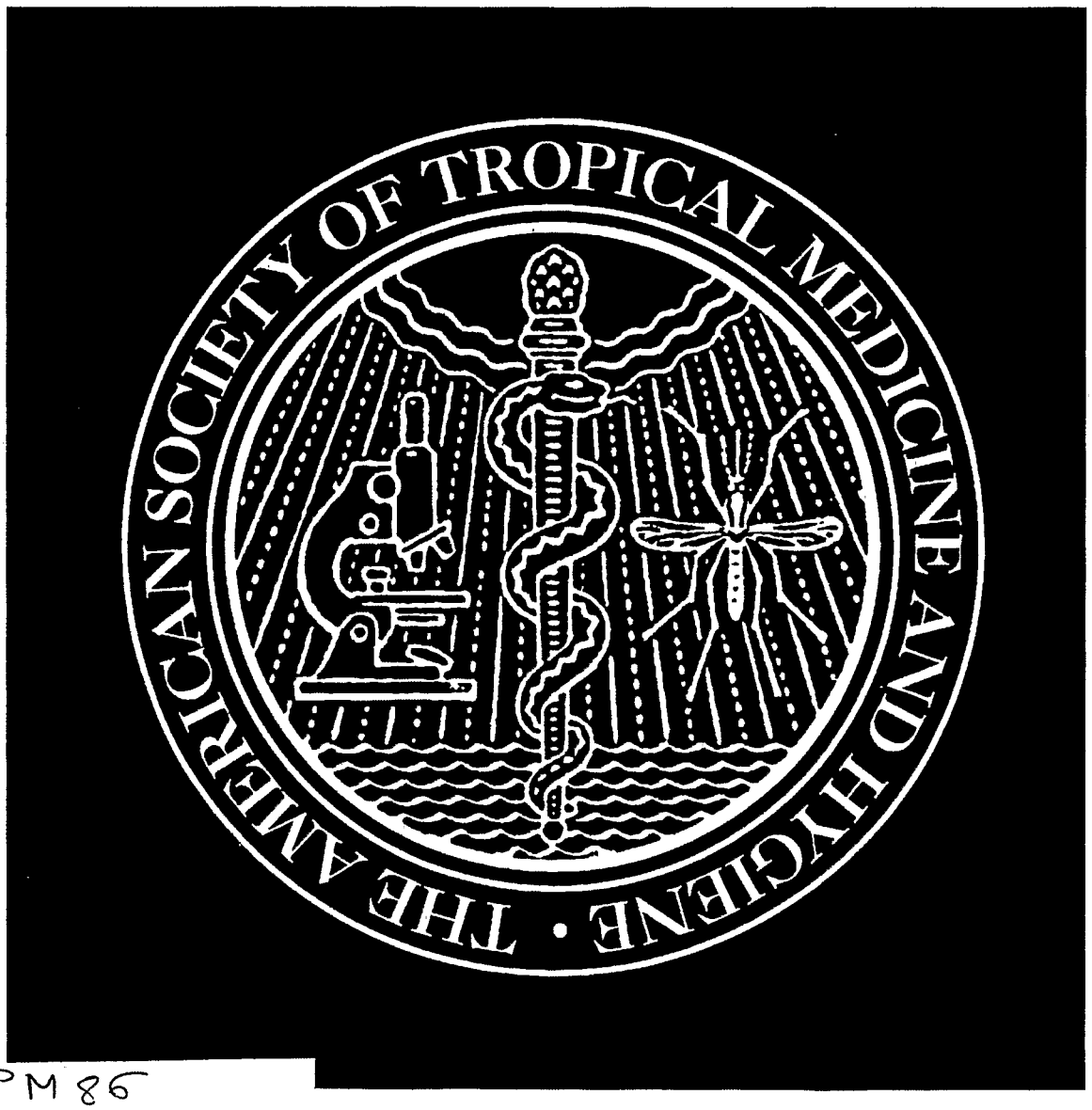

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