Am. J. Trop. Med. Hyg., 39(1), 1988, pp. 26–32 (87-281)

MONOCLONAL AND POLYCLONAL ANTIBODIES BOTH BLOCK AND ENHANCE TRANSMISSION OF HUMAN *PLASMODIUM VIVAX* MALARIA

J. S. MALIK PEIRIS,* SUNIL PREMAWANSA,† MAYA B. R. RANAWAKA,† PREETHI V. UDAGAMA,† YAMUNA D. MUNASINGHE,† MANJULA V. NANAYAKKARA,† C. PRIYANTHA GAMAGE,† RICHARD CARTER,‡ PETER H. DAVID,§ AND KAMINI N. MENDIS†

**Department of Microbiology, Faculty of Medicine, University of Peradeniya, Sri Lanka, †Department of Parasitology, Faculty of Medicine, University of Colombo, Kynsey Road, Colombo 8, Sri Lanka, ‡Department of Animal Genetics, University of Edinburgh, West Mains Road, Edinburgh EH9 3JN, Scotland, and §Unite d'Immunoparasitologie, Institut Pasteur, 25 Rue du Dr Roux, Paris 75724, France*

Abstract. Antibodies against gametes of the malarial parasite inhibit the development of the parasite in the mosquito and curtail the transmission of malaria. We now report that a monoclonal antibody against gametes of the human malaria pathogen *Plasmodium vivax* and antibodies induced during natural infections of *P. vivax* in humans which suppress infectivity of the parasites to the vector at high concentrations can, at lower concentrations, have the opposite effect and enhance the level of malaria infection in the mosquitoes. Infectivity enhancing effects of up to 12-fold were demonstrated when a transmission blocking monoclonal antibody and immune human sera were diluted, in some undiluted immune human sera, and in the sera of vivax malaria patients during convalescence after drug cure.

Extracellular gametes of malarial parasites emerge from their intraerythrocytic parental cells, the gametocytes, in the mosquito midgut following ingestion of malarious blood. Antibodies against these stages, when ingested together with malarial gametocytes in a bloodmeal, can suppress infectivity of the parasites to mosquitoes.[1–8] The presence of such antibodies in the vertebrate host has been termed malaria transmission blocking immunity. We report here that high concentrations of a monoclonal antibody (Mab) against gametes of the human pathogen, *Plasmodium vivax,* and also polyclonal antibodies induced during natural *P. vivax* infections in humans, suppress infectivity of the parasites in mosquitoes. In contrast, the same antibodies at lower concentrations have the opposite effect and considerably enhance the level of malaria infection in mosquitoes. These findings reveal unsuspected complexities in the biological properties of antibodies which, during natural infections, are likely to affect the transmission of *P. vivax* malaria and may complicate the development of malaria transmission blocking vaccines.

Accepted 21 December 1987.

MATERIALS AND METHODS

Parasites

P. vivax was obtained from patients with acute malaria who presented at the General Hospital, Colombo (GHC).[9] Colombo is a nonendemic area for malaria and the patients had contracted the disease on a visit to an endemic area. *P. vivax* parasites thus obtained from a single infection of a patient would be referred to as an "isolate."

Purification of female gametes

Gametocyte infected human blood was obtained following informed consent and female gametes were prepared by a method which has been previously described.[8] Briefly, gametocyte containing blood was washed in a suspended animation (SA) solution (10 mM Tris, 170 mM NaCl, and 10 mM glucose, pH 7.4), passed through a column of cellulose powder to remove white cells and subjected to conditions appropriate for gametogenesis in a gamete-releasing medium (SA solution containing 25 mM $NaHCO_3$ and 10% ox serum, pH 8.0) at room temperature for 30 min. Extracellular female ga-

metes were separated free of other parasite stages and host erythrocytes on a discontinuous gradient of Percoll.

Production of monoclonal antibodies

BALB/c mice were immunized with 3 doses, each containing 5×10^7 purified female gametes of *P. vivax*. The first 2 doses were given intraperitoneally, on days 0 and 30 followed by a final intravenous dose without adjuvant 4 days prior to the fusion. The first dose included *Bordetella pertussis* (0.5 ml of a 1:100 dilution of 10^8 reconstituted lyophilized organisms in phosphate buffered saline [PBS]) as an adjuvant. The spleen cells of an immunized mouse were fused with myeloma cells of the line P3-NS1/1-Ag 4-1(NS1) using 50% polyethylene glycol (1,500 M_r; Sigma Chemical Co., St. Louis, Missouri) according to the procedure described by Kohler and Milstein[10] and Galfre and others.[11] Supernatants from hybrid cell cultures were screened by the indirect immunofluorescent antibody test (IFT) on air dried female gametes of *P. vivax,* and selected hybrids were cloned twice by limiting dilution on thymocyte feeder layers. Cloned hybrids were injected intraperitoneally into pristane-primed mice to produce ascites fluids. Antibody was precipitated from hybridoma supernatants and ascites fluids with 45% ammonium sulfate and dialyzed against PBS before being tested in membrane feeding experiments.

Immune sera

Immune human sera were obtained from acute vivax malaria patients presenting at the GHC. Sera were decomplemented (at 56°C for 30 min) and stored at −20°C.

IgG was affinity purified from immune human sera and ammonium sulfate concentrated hybridoma culture supernatants using protein A-Sepharose (Sigma).[12]

Indirect immunofluorescence test

The IFT was performed as previously described[8, 13] using air dried female gametes and live unfixed gametes as antigen; the latter permitted the detection of antigens on the surface of extracellular gametes.

Assay for effect of antibodies on the infectivity of P. vivax *isolates to mosquitoes*

Four ml of blood were drawn with informed consent from gametocyte carriers among patients attending the GHC with acute infections of *P. vivax,* and diluted immediately in 40 ml of SA solution; in this solution gametocytes can be washed and maintained for several hours without being stimulated to undergo gametogenesis or losing their infectivity to mosquitoes. Dilutions of immune human sera (heat-inactivated) were made in normal human serum (type AB; heat-inactivated) (NHS). Dilutions of protein A purified Mabs or IgG from immune human serum were made in NS1 cell culture supernatants or IgG purified from NHS as appropriate, and mixed in equal proportions with NHS. (NHS was included in all feedings because whole serum appears to be necessary for infectivity.) The washed *P. vivax* parasitized blood cells were resuspended in antibody dilutions to a 50% hematocrit. For controls the blood was resuspended in either normal human serum or NS1 culture supernatants or protein A purified IgG from NHS mixed in equal proportions with NHS, as appropriate. These preparations were fed to *Anopheles tessellatus* from a laboratory bred colony through a water jacketed membrane feeding apparatus, circulating water at 40°C. The bloodfed mosquitoes were maintained for 7 days at 26–27°C and a relative humidity of 70%–80% and then dissected; their midguts were examined and the number of oocysts (products of malarial fertilization) counted. A minimum of 7 mosquitoes and an average of 12 were dissected for each experimental dilution and control feeding. The mean oocyst count per mosquito was used as an index of infectivity. "Infectivity" of a particular parasite isolate in a given serum or antibody was defined as the mean number of oocysts/gut of mosquitoes fed on that preparation.

SDS-polyacrylamide gel electrophoresis (PAGE) and Western blots

Purified female gametes were extracted in SDS sample buffer without reducing agents, and the polypeptides were electrophoretically separated on a 5%–15% SDS polyacrylamide gradient gel and transferred to nitrocellulose paper by electroelution. Strips of nitrocellulose with transferred gamete antigens were blocked by incu-

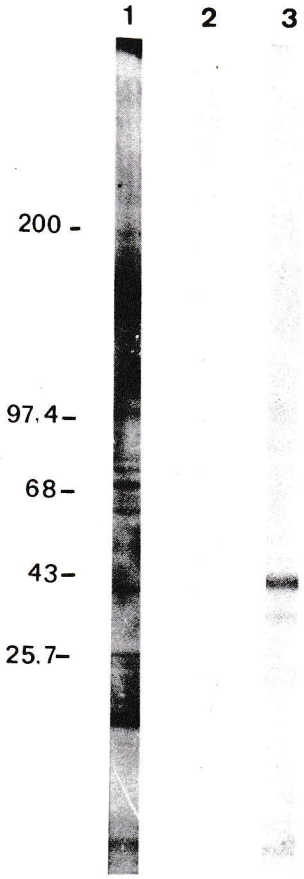

FIGURE 1. Western blot of *P. vivax* female gamete antigens extracted under nonreducing conditions and reacted with a transmission blocking immune human serum (lane 1), ammonium sulfate precipitated (×50 concentrated) culture supernatants of NS1 myeloma cells (lane 2), or of the hybridoma cell line producing transmission blocking Mab M1/12.D2/6H (lane 3).

bation in Tris buffered saline (pH 8.0) containing 5% milk power (Lakskim, Sri Lanka) and then incubated with antibody. After washing, the strips were incubated with a peroxidase-conjugated goat anti-mouse antibody (Bio-Rad, Richmond, California), and washed and reacted with a color development reagent containing 4-chloro-1-naphthol (Bio-Rad) to expose the sites of reaction of antibody with antigen.

RESULTS

Mab M1/12.D2/6H of gamma-1 isotype reacted with the surface of extracellular gametes of *P. vivax* by immunofluorescence. On Western blots this Mab reacted with two proteins of 42 and 36 kDa from extracts of gametes of *P. vivax* separated on SDS-PAGE under nonreducing conditions (Fig. 1). Mab M1/12.D2/6H when used in membrane feeding experiments in the form of ×50 concentrated hybridoma culture supernatants (dialyzed against PBS) suppressed the infectivity of most *P. vivax* isolates to <10% of controls. Protein A Sepharose purified IgG of this Mab at a concentration of 0.6 mg/ml also blocked the infectivity of *P. vivax* infected blood to *An. tessellatus* when presented in a membrane feeding apparatus (Fig. 2A). The 42 and 36 kDa proteins on the gametes of *P. vivax* thus represent antigenic targets for transmission blocking immunity.

When dilutions of the Mab M1/12.D2/6H (protein A purified) were tested in membrane feeding experiments, not only was the suppressive effect on infectivity of *P. vivax* to mosquitoes lost with increasing dilution, but in almost all experiments low concentrations of antibody enhanced infectivity by 2- to 4-fold above control levels, over a narrow range of antibody concentrations (Fig. 2A). In experiments where the antibody dilutions extended far enough, relative infectivity returned to control levels when diluted beyond the enhancing range (Fig. 2A).

The dual activity of suppression or enhancement of infectivity at different concentrations was not only found with the Mab but also with immune human sera. We had previously demonstrated the presence of transmission blocking antibodies in sera from acute *P. vivax* infections in adults in Sri Lanka.[14] In the present study we tested the effects of diluting 4 of these "blocking" sera (Table 1). All the sera enhanced infectivity of *P. vivax* when appropriately diluted, the maximum infectivity being 2- to 3-fold greater than in controls.

The enhancing effect of the human sera was shown to be antibody-mediated using immunoglobulins purified on protein A Sepharose from acute *P. vivax* infected human sera. These antibodies produced infections in mosquitoes when mixed with a *P. vivax* isolate whose gametocyte density was too low to produce an infection in the absence of the antibodies (Fig. 2B). Under similar circumstances mosquito infections were produced in the presence of dilutions of the Mab M1/12.D2/6H with an isolate of *P. vivax* which otherwise failed to infect.

Infectivity enhancement was also mediated by

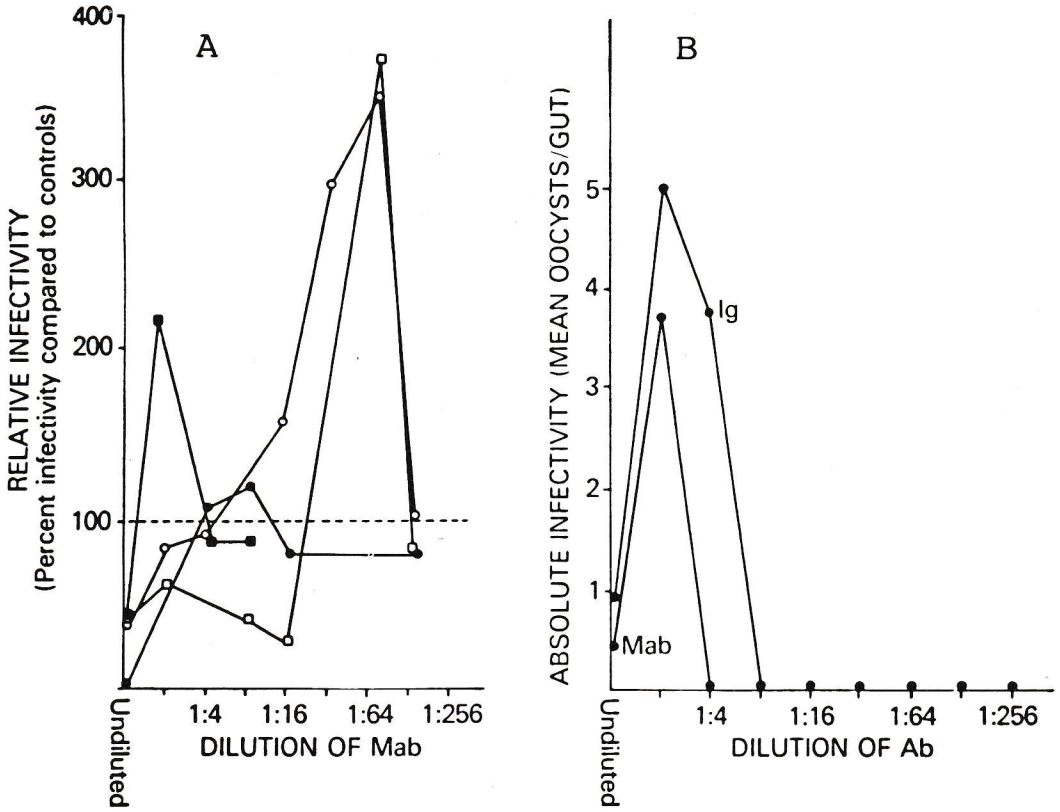

FIGURE 2. Antibody-mediated enhancement of infectivity of *P. vivax* to *An. tessellatus* mosquitoes. **A.** Dilutions of Mab M1/12.D2/6H purified on protein A Sepharose and reconstituted to a starting concentration of 0.68 mg/ml. Results with 4 different isolates of *P. vivax* are represented each by a different symbol. **B.** Dilutions of the protein A purified Mab (initial concentration 0.68 mg/ml) and protein A purified IgG from acute *P. vivax* infection serum (initial concentration 5 mg/ml), tested against 2 different isolates of *P. vivax*. In both these experiments the parasite isolates had zero infectivity in controls; infectivity in the presence of antibody is expressed as the number of oocysts per midgut.

some acute *P. vivax* sera in undiluted form. Among 40 *P. vivax* sera previously tested for their effects on infectivity of *P. vivax* to mosquitoes[14] 3 showed considerable enhancing activity (Table 2). The enhancement achieved by serum No. 458 was 13-fold greater than in the controls.

We have also studied the properties of undiluted sera taken from *P. vivax* patients during acute infection and convalescence. The anti-gamete antibody titers of these sera as measured by the IFT against air dried gametes[14] ranged from 1:160 to 1:640 during the acute infection and remained at these levels for about a month after drug cure. Thereafter the titers declined rapidly to reach low levels (1:80) in 4 months. In some patients the sera changed from infectivity blocking during acute infection to infectivity enhancing during convalescence (Fig. 3). In one patient the enhancing activity of the serum increased 12-fold over the control by 90 days post-acute infection and drug cure. The results suggest that these effects may be due to the enhancing activity of antibodies against the gametes or zygotes of *P. vivax*.

DISCUSSION

It is evident that antibodies both monoclonal and polyclonal in human immune sera which block malaria transmission to mosquitoes at high concentrations can at lower concentrations have the opposite effect on the parasite and enhance infectivity. As we have shown, antibodies can not only enhance the number of developing parasites in an infected mosquito but can also induce

TABLE 1

Infectivity enhancement effects of human immune sera at low concentrations

	% infectivity and (absolute infectivity as mean oocysts/gut)*					
Serum No.	E15-9	651	667		682	
Parasite isolate No.	M_r 690	M_r 727	M_r 675		M_r 723	M_r 710
Sera† / Serum dilution‡				Serum dilution‡		
NHS (control)	Neat	100.0 (46.5)	100.0 (7.1)	100.0 (22.5)	Neat	100.0 (90.1)	100.0 (13.1)
IHS	Neat	38.7 (18.0)	21.1 (1.5)	ND	1:16	0.48 (0.43)	ND
	1:2	234.1 (108.9)	ND	63.1 (14.2)	1:32	0.37 (0.33)	9.5 (1.25)
	1:4	ND	7.0 (0.5)	183.9 (41.4)	1:64	18.2 (16.4)	40.2 (5.3)
	1:8	165.5 (77.0)	42.2 (3.0)	283.2 (63.8)	1:128	ND	30.4 (4.0)
	1:16	ND	123.9 (8.8)	184.2 (41.5)	1:256	48.2 (43.4)	188.5 (24.8)
	1:32	ND	284.4 (20.2)	ND	1:512	112.5 (101.4)	277.4 (36.5)

* Dilutions of sera from 4 patients with acute *P. vivax* infections were tested in membrane feeding experiments, each against a single parasite isolate except serum No. 682 which was tested against 2 different parasite isolates.
† NHS = normal (nonimmune) human serum; IHS = immune human sera.
‡ For all sera except No. 682, the enhancing activity lay within the range of dilutions, neat–1:32, and for serum No. 682, 1:16–1:512.
ND = not done.

an otherwise noninfective isolate to infect mosquitoes (Fig. 2B). Since both suppression and enhancement of infectivities of *P. vivax* for mosquitoes were mediated by a single Mab at different concentrations, the same target epitope must be involved in both effects in this instance. In the human sera, however, the suppressing and enhancing effects could be mediated by antibodies against the same or different epitopes. The narrow range of antibody concentrations over which the enhancing effects were observed must reflect their mode of action but does not indicate what this mechanism might be. It was evident that different concentrations of the Mab were required to mediate infectivity enhancement for each of the 4 different isolates; this may be related to "strain" differences in the epitope recognized by the Mab.

TABLE 2

Enhanced infectivity to An. tessellatus *of* P. vivax *infected blood in the presence of autologous heat-inactivated serum from the infected patient*

	Infectivity (oocysts/gut)*	
Patient no.	In normal human serum	In patient's serum
422	7.8 ± 3.0	23.8 ± 4.9
458	5.8 ± 2.1	76.5 ± 16.9
462	30.6 ± 9.4	59.2 ± 12.1

* Mean ± SEM.

Implicit evidence of antibody-mediated enhancement of plasmodial development is shown in the results of previous studies. In a simian malaria system, it was demonstrated that *P. cynomolgi* infections from Rhesus monkeys with a past history of *P. knowlesi* were significantly more infectious to mosquitoes than from immunologically naive animals.[15] This was evident from both direct feeding of mosquitoes on infected animals as well as from membrane feeding experiments. However, preliminary experiments designed to implicate serum factors as a cause of this enhanced infectivity were unsuccessful. These observations provide a possible parallel to our findings. A comparable phenomenon has been described in another phase of the life cycle of the malaria parasite, viz., antibody-mediated enhancement of asexual blood infections of *P. berghei* in mice following immunization.[16] Passive transfer experiments indicated that the effect was mediated at least in part by antibody, and was concentration-dependent; whereas large amounts of passively transferred immune serum had no effect on a challenge infection, low doses exacerbated infection.

We now have preliminary but clear evidence that antibodies against gametes of two other malaria parasites *P. falciparum* and *P. cynomolgi,* in human and monkey immune serum, respectively, also enhance infectivity at low concentrations (data not shown). In a recent study Ponnudurai and others[17] have investigated the effects of diluting 2 transmission blocking anti-gamete and -zygote Mabs on the infectivity of *P. falciparum* gametocytes. Although the authors comment that ". . . both types of blockers lose their effect at low concentrations, the occasional slight increase in mean oocyst numbers lying within the normal range . . . ," it is noteworthy that the same trend of infectivity above control levels was observed in both their experiments. Even though significant enhancement of infectivity was not demonstrated in their study, it is possible that, in as much as the mechanisms of infectivity enhancement are still unclear, antibodies against only some gamete and zygote antigens mediate infectivity enhancement.

Enhancement of a biological function by antibodies is known in other situations: Arboviral replication in macrophages is enhanced by specific antibody[18–21] and is mediated by the macrophage Fc[22] and complement[23] receptors. Specific antibody increases the activity of the enzymes penicillinase[24] and phenylalanine hydroxylase[25] and of interferon;[26] it can also stimulate the growth and replication of thyroid cells.[27] Antibody-mediated enhancement of tumor growth and graft survival by the action of "blocking antibodies" has been recognized for many years. The mechanisms involved in these phenomena may not, however, be relevant to antibody enhancement of *P. vivax* gametes to mosquitoes. Some general possibilities may be proposed: antibodies cross-reacting with male and female gamete antigens could enhance fertilization efficiency; antibodies could induce conformational changes in surface molecules on the gametes to enhance fertilization or on the zygotes to stimulate subsequent growth and development; and antibodies could protect the parasites against antiparasitic factors in the mosquito bloodmeal.

Naturally occurring antibodies which either suppress[14] or enhance infectivity to mosquitoes during acute malarial infection could be expected to have important effects on the epidemiology of *P. vivax* malaria. The properties of these antibodies must be considered in efforts to develop transmission blocking vaccines.

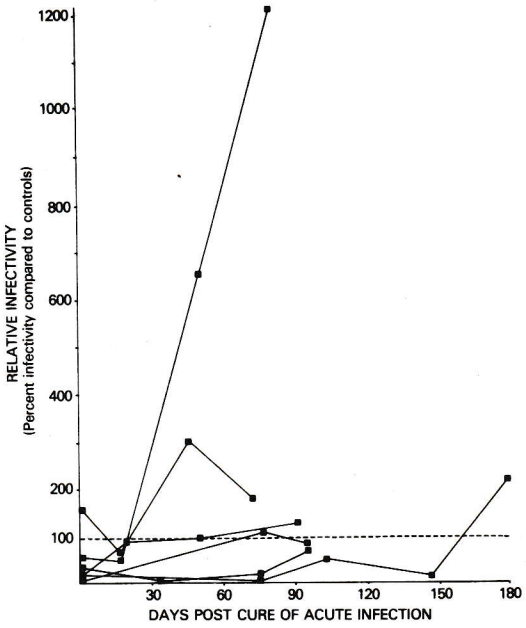

FIGURE 3. Effect of serum taken at different times after cure of *P. vivax* infections on infectivity of *P. vivax* to mosquitoes in membrane feeding experiments. Post-recovery sera from 6 different patients are represented.

ACKNOWLEDGMENTS

We thank S. Nanayakkara, Y. N. Y. de Silva, A. Jayasinghe, K. L. R. L. Perera, G. M. G. Kapilananda, and G. Gunasekera for their technical assistance, and W. Davis and B. Martin for editorial assistance. The Medical Superintendent, physicians, and nursing staff of the General Hospital, Colombo, and M. M. Ismail are acknowledged for their cooperation; as well as W. Abeywickreme, L. H. Miller, and S. M. Handunnetti for discussion.

This investigation received support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

REFERENCES

1. Gwadz RW, 1976. Successful immunization against the sexual stages of Plasmodium gallinaceum. *Science 193:* 1150–1151. UI:76271136
2. Carter R, Chen DH, 1976. Malaria transmission blocked by immunisation with gametes of the malaria parasite. *Nature 263:* 57–60. UI: 76267771
3. Mendis KN, Targett GA, 1979. Immunisation against gametes and asexual erythrocytic stages

of a rodent malaria parasite. *Nature 277:* 389–391. UI:81012039

4. Gwadz RW, Green I, 1978. Malaria immunization in Rhesus monkeys. A vaccine effective against both the sexual and asexual stages of Plasmodium knowlesi. *J Exp Med 148:* 1311–1323. UI:79069009
5. Kaushal DC, Carter R, Rener J, Grotendorst CA, Miller LH, Howard RJ, 1983. Monoclonal antibodies against surface determinants on gametes of Plasmodium gallinaceum block transmission of malaria parasites to mosquitoes. *J Immunol 131:* 2557–2562. UI:84034242
6. Rener J, Graves PM, Carter R, Williams JL, Burkot TR, 1983. Target antigens of transmission-blocking immunity on gametes of Plasmodium falciparum. *J Exp Med 158:* 976–981. UI: 83293233
7. Vermeulen AN, Ponnudurai T, Beckers PJ, Verhave JP, Smits MA, Meuwissen JH, 1985. Sequential expression of antigens on sexual stages of Plasmodium falciparum accessible to transmission-blocking antibodies in the mosquito. *J Exp Med 162:* 1460–1476. UI:86035933
8. Munesinghe YD, Mendis KN, Carter R, 1986. Anti-gamete antibodies block transmission of human vivax malaria to mosquitoes. *Parasite Immunol 8:* 231–238. UI:86258716
9. Fonseka J, Mendis KN, 1987. A metropolitan hospital in a non-endemic area provides a sampling pool for epidemiological studies on vivax malaria in Sri Lanka. *Trans R Soc Trop Med Hyg 81:* 360–364. UI:88071638
10. Kohler G, Milstein C, 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature 256:* 495–497. UI:76010727
11. Galfre G, Howe SC, Milstein C, Butcher GW, Howard JC, 1977. Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature 266:* 550–552. UI:77171275
12. Hudson L, Hay FC, 1980. Preparation of IgG on protein A-Sepharose. in *Practical immunology.* Oxford: Blackwell Scientific Publications, 220–222. UI:8102006
13. Mendis KN, David PH, Hommel M, Carter R, Miller LH, 1983. Immunity to malarial antigens on the surface of Plasmodium falciparum-infected erythrocytes. *Am J Trop Med Hyg 32:* 926–930. UI:84021559
14. Mendis KN, Munesinghe YD, de Silva YN, Keragalla I, Carter R, 1987. Malaria transmission-blocking immunity induced by natural infections of Plasmodium vivax in humans. *Infect Immun 55:* 369–372. UI:87107900
15. de Arruda-Mayr M, Cochrane AH, Nussenzweig RS, 1979. Enhancement of a simian malarial infection (Plasmodium cynomolgi) in mosquitoes fed on rhesus (Macaca mulatta) previously infected with an unrelated malaria (Plasmodium knowlesi). *Am J Trop Med Hyg 28:* 627–633. UI:79229503
16. Jerusalem C, Weiss ML, Poels L, 1971. Immunologic enhancement in malaria infection (Plasmodium berghei). *J Immunol 107:* 260–268. UI:71234512
17. Ponnudurai T, van Gemert GJ, Bensink T, Lensen AH, Meuwissen JH, 1987. Transmission blockade of Plasmodium falciparum: Its variability with gametocyte numbers and concentration of antibody. *Trans R Soc Trop Med Hyg 81:* 491–493. UI:88071669
18. Halstead SB, O'Rourke EJ, 1977. Antibody-enhanced dengue virus infection in primate leukocytes. *Nature 265:* 739–741. UI:77171190
19. Halstead SB, O'Rourke EJ, 1977. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. *J Exp Med 146:* 201–217. UI:77208841
20. Halstead SB, O'Rourke EJ, Allison AC, 1977. Dengue viruses and mononuclear phagocytes. II. Identity of blood and tissue leukocytes supporting in vitro infection. *J Exp Med 146:* 218–229. UI:77208842
21. Peiris JS, Porterfield JS, 1979. Antibody-mediated enhancement of Flavivirus replication in macrophage-like cell lines. *Nature 282:* 509–511. UI:80054771
22. Peiris JS, Gordon S, Unkeless JC, Porterfield JS, 1981. Monoclonal anti-Fc receptor IgG blocks antibody enhancement of viral replication in macrophages. *Nature 289:* 189–191. UI: 81099007
23. Cardosa MJ, Porterfield JS, Gordon S, 1983. Complement receptor mediates enhanced flavivirus replication in macrophages. *J Exp Med 158:* 258–263. UI:83240463
24. Pollock MR, Fleming J, Petrie S, 1967. Effect of specific antibodies on the biological activities of wild type bacterial penicillinases and their mutationally altered analogues. in Cinader B, ed. *Antibodies to biologically active molecules.* Oxford: Pergamon Press 139–152. UI:0117055
25. Choo KH, Jennings IG, Cotton RH, 1981. Comparative studies of four monoclonal antibodies to phenylalanine hydroxylase exhibiting different properties with respect to substrate-dependence, species-specificity and a range of effects on enzyme activity. *Biochem J 199:* 527–535. UI:82182053
26. Nagano Y, Koima Y, Oda M, Kin T, Shirasaka M, Haneishi T, 1965. Inactivation du facteur inhibiteur du virus par l'antiserum. *C R Soc Biol (Paris) 159:* 280–283.
27. Drexhage HA, Bottazzo GF, Doniach D, Bitensky L, Chayen J, 1980. Evidence for thyroid growth stimulating immunoglobulin in some goitrous thyroid diseases. *Lancet 2:* 287–292.