RELIABLE EVIDENCE OF INVOLVEMENT OF THE KININ SYSTEM IN MOUSE MALARIA

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The pharmacology of bradykinin has enticed investigators to prove the involvement of the kinin system in various pathological states, including protozoan diseases.

In *Plasmodium knowlesi* malaria of rhesus monkeys, the significance of the kinin system was reported by British investigators, who showed that plasma kininogen of the infected primates was almost depleted near death, in spite of failure of the detection of free kinin in the plasma (1, 2). In addition, the increase of kallikrein contents and kininase activity in plasma were also reported in the infected monkeys (2, 3).

On the other hand, Urbanitz, et al. claimed that the reduced concentration of plasma kininogen did not mean directly the involvement of the kinin system in various types of shock, because the reduction of plasma kininogen levels was simultaneously accompanied with the reduction of plasma protein concentration in shocks (4, 5).

As it is known that plasma protein concentration decreases gradually with the increased degree of parasitaemia in the monkey malaria (6), the apparent reduction of plasma kininogen in the infection mentioned above may be one of the features of the general prostration without true consumption of kininogen and release of kinin. Therefore, the reduction of kininogen in malaria must be re-examined in these respects.

The present paper reports, in the severe cases of mice infected with *Plasmodium berghei* (NK 65), the success of the detection of free kinin in venous blood and the real consumption of plasma kininogen. The latter finding was supported by the reduction of the concentration expressed in $\mu\text{g}/\text{mg}$ protein or globulin. Its pathophysiological significance is also discussed.

MATERIALS AND METHODS

Infections

Plasmodium berghei (NK 65) was used for the infection of malaria to mice. The strain, isolated by Yoeli (7), was kindly supplied in September 1969 by Department of Bacterial Infection, The Institute of Medical Science, University of Tokyo. The maintenance for experiments was done by i.p. inoculation of mice usually every five days. For the

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infection experiments, healthy female mice of dd-strain, weighing 19–21 g, were inoculated i.p. with 0.1–0.2 ml of infected mouse blood, diluted with saline, which contained approx. 10^7 of parasitized red cells. Infected mice were housed at approx. 25°C. As the infection was fatal on around day 7 of inoculation, blood samples were collected from mice on the day 7. Day 1 equals 24 hr after inoculation. Non-infected control mice were fed parallelly in the same condition and used for experiments on the same day as the infected mice.

Parasite counts

This was done on the blood thin films obtained from the collected blood of the infected animals. The degree of parasitaemia is expressed in percentage of the infected erythrocytes to thousand non-parasitized red cells.

Blood collection

Infected and non-infected mice were anesthetized with pentobarbital sodium (50 mg/kg i.p.). In the initial few experiments, blood samples were collected by cardiac puncture using a disposable needle and a plastic syringe. Because of haemolysis, however, in most other cases this procedure was replaced by collection from the carotid artery through a thin cannula (PE-10). Blood was drawn slowly and carefully, by a plastic syringe moistened with heparin (250 u/ml). Haemolysis was avoided carefully and haemolyzed plasma was discarded. 0.8–1.2 ml of the blood was obtained from the carotid artery using this procedure. The blood placed in small plastic tubes was centrifuged at 0°C for 30 min at 4000 G. The plasma obtained was used for the determination of kininogen, protein, electrophoresis and kininase activity. Haematocrit values were determined with 0.05 ml of blood by Kokusan microhaematocrit centrifuge. Contacts with glass as well as negative surfaces were carefully avoided.

Bioassay of kinin

The isolated rat uterus preparation was used for determination of free kinin, kininogen and kininase activity. Virgin rats weighing 120–200 g were injected i.p. and s.c. with 7.5 mg of hexestrol (Hexron, Teikoku Zōki) 12–24 hr before assay. One horn of the separated uterus was suspended at 28°C in 5 ml organ bath filled by aerated Munsick solution in the presence of 10^{-7} g/ml of 2-bromolysergic acid diethylamide (BOL, Sandoz). The contractions of the uterus were recorded using a frontal writing lever. Synthetic bradykinin (Peptide Centre, Institute for Protein Research, Osaka University, Osaka) was used as a standard. The contact time for each sample was 90 sec, and the interval between tests was 4 to 5 min. Munsick solution contained NaCl 114.0 mM, KCl 6.2 mM, CaCl_2 0.5 mM, NaH_2PO_4 1.0 mM, NaHCO_3 30.0 mM, glucose 0.5 g in distilled water 1.0 l (8).

Extraction of kinin

Blood was collected from the jugular vein of infected and non-infected mice in the same way as the method used for blood collection from the carotid artery. A half ml of blood was almost the total amount which could be collected from jugular vein of mice using this method. The volume of the venous blood was ejected into 15 ml of chilled 80% (v/v) ethanol in polyethylene tube and kinin was extracted according to the method reported by

Brocklehurst and Zeitlin (9). The dried residues were stored at -20°C and dissolved in 0.5 ml of warm Munsick solution immediately before assay. Because of the small amount of venous blood (about 0.5 ml), the sensitivity of the rat uterus was increased by reducing the load on the uterus, so that 0.1 ng/ml of bradykinin in the organ bath contracted the uterus by 1–30 mm on the record.

The four recovery experiments were made with 1 μg of bradykinin, added to 80% ethanol at the same time as addition of blood from control mice. The values obtained were 57.0, 52.0, 50.0 and 56.0%. The values of recovery for four other experiments with human blood samples were 69.3, 78.4, 64.6 and 70.7% (mean 70.8%).

Total kininogen

The determination of plasma kininogen was done according to the method reported by Diniz and Carvalho (10). Acidified, denatured plasma of mice were incubated for 30 min with trypsin (Nutritional Biochemical Corporation, U.S.A.) and released bradykinin was extracted with alcohol. The dried residues were stored at -20°C until use. Kinin in 0.05 ml of sample dissolved in 4 ml of Munsick solution was assayed on the rat uterus. Recently, it was reported that the method also produced potentiator(s) of bradykinin (11, 12). Although this was confirmed in our laboratory (to be published), kininogen determination in these experiments was performed by the original method with the following special attentions: The volume of Munsick solution required to dissolve dried residues and samples to be put into the organ bath was restricted to 4 ml and 0.05 ml respectively.

Plasma rininase activity

Synthetic bradykinin (1 μg) was incubated with 0.05 ml of plasma from normal and infected mice at 37°C in the presence of soy bean trypsin inhibitor (0.2 mg) (Calbiochem, U.S.A.). The final volume of the incubation mixture was adjusted to 2 ml with 0.1 M phosphate buffer (pH 7.4). 0.05 ml of the incubation mixture was removed and assayed on the rat uterus at 5 min intervals for residual kinin activity. Kininase activity was expressed in terms of the half-life of bradykinin.

Plasma protein determination

The method reported by Lowry et al. (13) was used in most experiments, but, at the beginning of the experiment, the determination was done by rellactometer. As protein values determined by both methods were not significantly different within the values in the experiments, all values were incorporated into the figures.

A/G ratio of plasma protein by electrophoresis

To obtain albumin-globulin ratio with the limited volume of the mouse plasma, electrophoresis on cellulose acetate membrane (Sartorius Membranfilter GmbH, Germany) was used. Four μl of plasma was used for fractionation. Both albumin and globulin fractions were stained and extracted with 3.5 ml of 0.01 N NaOH. The colour in NaOH solution was read at 510 m μ by spectrophotometer. Unstained part of the membrane was used for a blank. A/G ratio was calculated from extinctions of both albumin and globulin. The plasma concentrations of both fractions were calculated from the total protein concentration.

Measurement of blood pressure of mice

Infected and non-infected control mice were anesthetized with pentobarbital sodium (50 mg/kg i.p.). Systemic arterial pressure was measured by high pressure transducer (Nihon Kohden, MPU-0.5) connected to the thin cannula (PE-10) inserted to the carotid artery of the mice. Respiratory movements of the thorax were traced by strain-gage transducer (Nihon Kohden, SB-1T) and both were recorded on an ink-writing oscillograph.

In relation to the blood pressure experiment, bradykinin potentiator C was used for proof of the circulating kinin. The peptide is one of five bradykinin potentiators isolated from the venom of a snake (*Agkistrodon halys blomhoffii*) and was kindly supplied by Dr. H. Kato and Prof. T. Suzuki, Institute for Protein Research, Osaka University.

RESULTS

Infection

Mice infected with *Plasmodium berghei* (NK 65) showed no appreciable pathological phenomena until day 3 of infection. Around day 5 after infection, pathological features became evident gradually: Body weight stopped increasing or rather it decreased, and the mice showed dullness in action, piloerection, decreased food intake, closed eyelids, pale and cold skin, particularly in tail and ears, and anemia. General prostration was evident even macroscopically. Some mice died from around day 6 of infection.

FIG. 1. Increase in the degree of parasitaemia of three mice in the time course after inoculation.

Three mice were inoculated i.p. with approx. 10^7 parasitized red cells. Ordinate, the degrees of parasitaemia (%); abscissa, days after inoculation.

FIG. 2. Distribution of parasitaemia in seventy-one mice on day 7 of infection.

Ordinate, number of mice; abscissa, the degrees of parasitaemia (%).

Fig. 1 depicts the results from pilot study, with three mice in which the degrees of parasitaemia were followed after inoculation. The parasite count for the pilot study was made with 200 red cells on the film of blood taken by pricking tail. Parasitaemia did not increase before day 3 of infection, whereas it increased suddenly between day 5 and 6, and further from day 7 onwards. The parasitaemia of 71 infected mice on day 7 was 30–40% at the peak and was distributed evenly to both sides from 4 to 72%, as shown in Fig. 2. In the following experiments, results were grouped tentatively into two, so that mice with parasitaemia of less than 30% were grouped as the mild cases, whereas those with more

FIG. 3. Relationship of kininogen, body weight, haematocrit and total plasma protein to parasitaemia in the infected (■) and the non-infected, control (●) mice. a) kininogen ($\mu\text{g/ml}$); b) body weight (%); c) haematocrit (%); d) plasma protein (mg/ml). Abscissa, the degrees of parasitaemia (%); Cont, control mice.

than 30% the severe cases.

Total kininogen

Fig. 3 (a) shows the total plasma kininogen contents per ml plasma of infected and non-infected control mice, plotted against the parasitaemia of each mouse. The total kininogen contents of 71 infected mice decreased with the increased degree of parasitaemia. The kininogen in the severer cases was obviously lower, compared with those of 51 non-infected mice. However, the decreased concentration of the kininogen of the infected mice may not always mean the consumption of the kininogen, because plasma protein as well as body weight and haematocrit of the infected mice decreased with the increased degree of

FIG. 4. Changes of total plasma protein and kininogen in the control mice and in the mild and the severe cases of the infected mice.

Mice with parasitaemia below 30% were grouped as mild cases, whereas those with above 30% as severe cases. The kininogen concentrations per ml plasma ($\mu\text{g}/\text{ml}$) were significantly lower in the infected mice than in the control, as were the total plasma protein concentrations (mg/ml) in the infected mice. The numbers of mice used are shown in parenthesis. Mean $\pm$ standard error. ** $p < 0.01$.

FIG. 5. Kininogen contents expressed as per mg protein in the control and the infected mice.

The kininogen content was lower only in the severe cases (** $p < 0.01$) than in the control group. The numbers of mice used were 42, 21, 40 for the control, mild and severe cases respectively. KGN, kininogen. Mean $\pm$ standard error.

parasitaemia as shown in Fig. 3 (b)(c)(d). In fact, although mean 3.8% increase of body weight was shown in all the control mice for 7 days, the infected animals showed a marked decrease in body weight, according to the severity of the disease. Haematocrit was also reduced in the infected group. Plasma protein concentration decreased with the increased parasitaemia as well, although the slope was not so steep. The relationship between the total plasma kininogen and the plasma protein is illustrated in Fig. 4. The kininogen contents of the non-infected, control mice were compared with two groups of the infected mice; the mild cases (less than 30% in parasitaemia) and the severe cases (more than 30%). The total kininogen of the severe cases (mean $\pm$ S.E., 6.00 ± 0.27 μ g/ml with 45 mice) was significantly lower ($p < 0.01$) than that of the control, non-infected mice (8.92 ± 0.16 μ g/ml with 51 mice). The difference between the mild cases (7.86 ± 0.31 μ g/ml with 26 mice) and the control mice was also significant ($p < 0.01$). Plasma protein concentrations in the control, mild and severe groups were 52.5 ± 0.9 , 48.2 ± 1.4 and 44.7 ± 1.2 mg/ml of plasma respectively (mean $\pm$ S.E.) and the latter two were significantly lower than the former ($p < 0.01$). From these results, the possibility cannot be denied that the reduction of the total plasma kininogen was only a part of the reduced concentration of the plasma protein, without any release of kinin. If so, the kininogen concentration per mg protein of each animal must be non-significant among three groups. Results are illustrated in Fig. 5. The value of the severe group (0.133 ± 0.006 μ g/mg) was definitely lower ($p < 0.01$) than that of the control (0.169 ± 0.004 μ g/mg) and the mild group (0.159 ± 0.007 μ g/mg; $p < 0.01$), but there was no significant difference between the mild group and the control group ($p > 0.2$). Therefore it must be concluded that the true consumption of kininogen occurred in the severe group, whereas plasma kininogen was not consumed in the mild group, even if the kininogen per ml plasma was reduced significantly.

Considering the general prostration of the infected mice, the reduced concentration of protein in plasma may be due to the decreased amount of albumin and not to that of globulin in which plasma kininogen is present. Albumin-globulin ratios obtained are shown in Table 1. Although the significant reduction was observed in the severe group ($p < 0.01$), A/G ratio of the mild group was not significantly different from that of the control group. The concentrations of albumin and globulin in three groups, calculated from the total protein concentration, are illustrated in Fig. 6.

Changes of the protein concentration were very similar to those in Fig. 4, except the

TABLE 1. Albumin-globulin ratios of plasma protein in the control and the infected mice.

	Control	Infected	
		Mild	Severe
Parasitaemia		below 30%	above 30%
No. of animals	18	10	15
A/G ratio	1.40 ± 0.02	1.33 ± 0.06	$0.95 \pm 0.14^{**}$

Mean $\pm$ standard error

** significantly lower than other two ($p < 0.01$)

slightly higher value of the severe group (48.4 ± 1.2 mg/ml) in this series than that in Fig. 4 (44.7 ± 1.2 mg/ml). Albumin concentrations, as can be supposed, gradually lowered, in a reflection of the increased degrees of parasitaemia, and the mean values $\pm$ S.E. obtained for the control, mild and severe groups were 32.3 ± 0.8 , 27.9 ± 1.4 and 23.4 ± 0.7 mg/ml respectively. This means that most of the total protein reduction during the process of the infection can be explained by the albumin reduction. On the contrary, the pattern of globulin concentration was somewhat different and interesting: Namely, that below 30% of parasitaemia (21.0 ± 0.8 mg/ml) decreased significantly ($p < 0.05$) from that of control (23.1 ± 0.4 mg/ml), but the globulin concentration increased again in the parasitaemia above 30% (25.0 ± 0.8 mg/ml; $p < 0.05$) and the value exceeded significantly that of the control group. The increased value of globulin in the severe group may explain partly the higher value of the total protein (Fig. 6) as well as the marked decrease of A/G ratio in this series (Table 1). Considering the patterns of albumin and globulin, recalculation of kininogen

FIG. 6. Changes of total plasma protein, albumin and globulin in the control and the infected mice.

The values in both cases of the infected mice were compared with those in the control cases. The numbers of mice used in the control, mild and severe cases were 18, 10, 15 respectively. Mean $\pm$ standard error. * $p < 0.05$ ** $p < 0.01$.

FIG. 7. Kininogen contents per mg albumin (a) and globulin (b) in the control and the infected mice.

The kininogen contents per mg albumin were not different among the three groups. The kininogen content per mg globulin in the severe cases decreased clearly (** $p < 0.01$), whereas that in the mild cases was not different from that in the control. The numbers of mice used are the same as in Fig. 6. Mean $\pm$ standard error. KGN, kininogen.

TABLE 2. Kininogen amounts in whole plasma and albumin and globulin fractions.

No. of experiments	I	II	III
Whole plasma ($\mu\text{g/ml}$)	5.20	5.60	5.60
Albumin ($\mu\text{g/ml}$)	0.00	0.00	0.00
Globulin ($\mu\text{g/ml}$)	3.79	4.00	4.24
Recovery (%)	72.9	71.4	75.7

The values for the whole plasma were obtained without running after application of 24 μl of plasma on the cellulose acetate membrane. Other 24 μl of the same plasma was fractionated on the membrane. Albumin and globulin were used for kininogen determinations. Recovery means the percentage of kininogen in globulin fraction against that of whole plasma.

contents per mg globulin might clarify whether true consumption of kininogen occurred or not, since kininogen is present in globulin fraction and its amount per mg globulin must be reduced after consumption.

As shown in Fig. 7 (b), the kininogen content per mg globulin was reduced significantly in the severe group ($0.245 \pm 0.015 \mu\text{g/mg}$; $p < 0.01$), compared with that in the control group ($0.376 \pm 0.011 \mu\text{g/mg}$). Again that of the mild group was not reduced significantly ($0.362 \pm 0.025 \mu\text{g/mg}$; $p > 0.25$). Consequently, it could be concluded that the kininogen was not consumed in the mild group in any aspect. It is plausible that the kininogen contents per mg albumin in the mild and the severe groups were not significantly different from the kininogen of the control group, as shown in Fig. 7 (a).

In addition, kininogen was not detected from the albumin fraction after separation by electrophoresis on the cellulose acetate membrane, whereas kininogen was present in the globulin fraction, as shown in Table 2. The values of recovery were 72.9, 71.4, 75.7%, when compared with the whole plasma which was applied on the membrane and was eluted by 0.01 N NaOH without running on the membrane.

From the results of the kininogen experiments, the following can be concluded: a) In the severe cases, the kininogen was consumed even in consideration of protein or globulin concentrations in plasma. b) In the mild cases, in spite of the significant reduction of kininogen per ml plasma, the kininogen did not appear to be consumed, when expressed as $\mu\text{g/mg}$ protein or globulin.

Free kinin

In order to know whether or not reduction of kininogen in plasma was really coupled with kinin release, free kinin in venous blood was studied.

Free kinin contents in plasma of infected and non-infected mice were plotted against the degrees of parasitaemia, as shown in Fig. 8. In 6 non-infected mice, the amount of free kinin were so small that all values were less than 1 ng/ml of blood. In the mild group of the infected mice (4 cases), kinin was detected in the same amounts as the control group. This finding coincided well with the conclusion of the kininogen experiments, that kininogen was not consumed. On the contrary, the venous blood from mice of the severe group (18 cases) contained much larger amounts of kinin. The values were scattered from 1 to

98 ng/ml blood, but a definite relationship between the amounts and the degrees of parasitaemia did not seem to be present, in spite of the tendency for the kinin amounts to increase with the severity of parasitaemia. As the findings were in good agreement with the results of the kininogen experiments, the reduction of kininogen contents in the severe group was concluded to be the real consumption in coupling with kinin release.

Kininase activity

Kininase activity was determined with plasma of mice in the control (9 cases), mild (7 cases) and severe groups (14 cases), as shown in Table 3. When the activity of kininase in plasma was expressed as the half-life of bradykinin added to the incubation mixture, that of the control mice ranged from 3.7 to 5.7 min and the mean value $\pm$ S.E. was 4.7 ± 0.2 min. The activities in mice of the mild and the severe infection were 2.0 ± 0.6 min and 1.4 ± 0.2 min respectively (mean $\pm$ S.E.). Both activities were increased significantly ($p < 0.01$), compared with the activity in the control mice. There was, however, no difference between the two groups of infected mice.

Systemic arterial pressure

Since the involvement of the kinin system was proved in the severe cases of the malarial infection in the present paper, the pathophysiological significance of the system was studied.

Systemic arterial pressure of the infected and the non-infected mice was determined and the values obtained are plotted against the degrees of parasitaemia (Fig. 9). The arterial pressure in the control mice was 80.2 mmHg as average, ranged from 60 to 94 mmHg with 13 mice, whereas the arterial pressure of the infected mice was scattered from 35 to 65

FIG. 8. Free kinin in venous blood of the control and the infected mice.

Ordinate, kinin extracted from the venous blood (ng/ml blood); abscissa, the degrees of parasitaemia (%). The amounts of kinin less than 1 ng/ml were plotted as 1 ng/ml of kinin. Closed circle, control (cont); open circle, infected.

Table 3. Plasma kininase activity of the control and the infected mice.

	Control	Infected	
		Mild	Severe
Parasitaemia		below 30%	above 30%
No. of animals	9	7	14
Half-life of bradykinin (min)	4.7 ± 0.2	$2.0 \pm 0.6^{**}$	$1.4 \pm 0.2^{**}$

Kininase activity was expressed as half-life of bradykinin.

Mean $\pm$ standard error. ** significantly higher activity than that in the control ($p < 0.01$).

mmHg with the mean value of 51.4 mmHg. Though the apparent relationship between the arterial pressure and the degrees of parasitaemia did not seem to be present, it could be said within the limits of this experiment that the infected mice showed hypotension. This finding might suggest that kinin extracted from the venous blood of the infected mice decreased the systemic arterial pressure. However, in order to explain the hypotension with extracted kinin, other proofs on the presence of the circulating kinin should be provided. In the present investigation, a preliminary experiment was done with bradykinin potentiator C.

When the circulating kinin was present, further hypotension could be expected in the infected mice after intravenous injection of potentiator C. The slow and careful injection of 0.3 μ g or 3 μ g/10g body weight of potentiator C through jugular vein of mice produced no hypotension not only in the control mice, but also in the infected mice with parasitaemia between 16.0 and 60.4%. The amount of potentiator C was sufficient so that it potentiated the hypotensive responses to 0.1 μ g/10g of bradykinin by

FIG. 9. Systemic arterial pressure under anesthesia in the control and the infected mice.

Ordinate, systemic arterial pressure (mmHg); abscissa, the degrees of parasitaemia (%). The numbers of mice used were 13 for the control and 11 for the infected. *Cont.*, control.

TABLE 4. Systemic arterial pressure and hypotensive responses to bradykinin in the control and the infected mice.

Control				Infected			
BP (mmHg)	Response to BK (decrease of BP, mmHg)		Parasi- taemia (%)	BP (mmHg)	Response to BK (decrease of BP, mmHg)		
	before P	after P			before P	after P	
87.5	37	—	34.9	45	5	10	
60	22.5	—	45.0	45	27.5	30	
80	25	—	16.0	45	17.5	12.5	
65	12.5	57.5	34.9	50	12.5	10	
75	30	57.5	27.8	50	12.5	7.5	
Mean	73.5	25.4	57.5	47.0	15.0	14.0	

BP, systemic arterial pressure;
BK, bradykinin (0.1 μ g/10 g i.v.);
P, bradykinin potentiator C (0.3 μ g/10 g i.v.)

3 to 5 times in the control mice. It was found, however, that this method was not satisfactory, because bradykinin injected intravenously was not enhanced after potentiator C in the cases of the infected mice. As shown in Table 4, the hypotensive responses to 0.1 $\mu\text{g}/10\text{g}$ of bradykinin were less in the infected mice than in the control and potentiator C did not potentiate the responses.

DISCUSSION

According to the pilot experiments with three mice inoculated with approx. 10^7 parasitized red cells, parasitaemia began to increase on day 3 after inoculation and the parasitaemia became suddenly severe between day 5 and 6. The results are not in agreement with the report given by Tella and Maegraith (14), that parasitaemia of mice increased gradually from day 1 after infection with 2×10^7 parasites/g body weight. The difference may be due to the strain of mice and parasite as well as the amount of the parasite used. Results in this paper however coincided with those in the other report (15).

Although it is preferable to follow biochemical alterations in plasma with one animal in the lapse of time after infection, experiments with mice make the realization impossible. Therefore, on day 7 of the infection, blood was drawn from mice under pentobarbital sodium anesthesia. Parasitaemia, haematocrit, protein, kininogen and A/G ratio were assessed with blood from one animal.

In these experimental conditions, the possibility of the involvement of the kinin system was studied. The best way to prove the involvement is to detect free kinin in the pathological states. Free kinin was definitely detectable in the severe cases of malaria, as shown in Fig. 8. For free kinin, venous blood was collected, as 78% of circulating bradykinin disappears in a single passage of the pulmonary circulation in cats (16). Failure to detect kinin in the malaria-infected monkeys may be partly due to the utilization of the arterial blood (1).

Since the extracted substance contracted the isolated rat uterus in the presence of an anti-serotonin agent (BOL), relaxed the isolated rat duodenum and caused hypotension in rats, the substance could be considered as kinin. Furthermore, according to Brocklehurst and Zeitlin (7), recovery of other smooth muscle stimulants extracted by this method were reported in mean $\pm$ standard deviation as follows; histamine, $0.4 \pm 0.8\%$; 5-hydroxytryptamine, $3.2 \pm 0.9\%$; K⁺, $6.2 \pm 2.1\%$. The recovery of bradykinin was $81.5 \pm 8.0\%$ in their experiments with human blood, whereas in our experiments with human blood 70.8% was the average. Even considering the values with human blood in the present experiment, 50–57% recovery with mouse blood was low. Although the higher kininase activity of mouse plasma may be a conceivable explanation, addition of o-phenanthroline or acidification of 80% ethanol at pH 5 did not improve the degree of recovery.

Under these experimental conditions, the extracted amount of free kinin from blood of the control, non-infected mice was less than 1 ng/ml blood. In mild cases with less than 30% parasitaemia, the amount did not exceed that in the control mice, and this result casts doubt on the possibility of the involvement of the kinin system in the mild

cases of the malarial infection in mice. On the contrary, in severe cases the values of free kinin extracted were scattered but definitely increased, though clear relationship between the kinin amounts and the degrees of parasitaemia did not seem to be present. Because of the total exanguination from the jugular vein, artificial formation of kinin during collection, may be suspected but the careful avoidance of contact with the negative surface and the detection of a minimal amount of kinin in the control and mild groups dispels any suspicion.

The involvement of the kinin system only in the severe cases was ensured even more by the consumption of kininogen in plasma. The kininogen concentrations per ml plasma were reduced in conjunction with the increased degrees of parasitaemia (Fig. 3), in such a way that the concentrations were significantly lower not only in the severe cases but also in the mild cases, as far as those were expressed as μg per ml plasma (Fig. 4). The reduction of the plasma protein contents deserves serious consideration, however, as pointed out by Urbanitz, Sailer and Habermann (4, 5). In the present experiments, calculation of kininogen contents per mg protein in plasma revealed a clear reduction of kininogen in the severe group, but erased the difference of the kininogen concentrations between the control and the mild groups.

On the latter finding, it may be claimed that the kininogen contents should be expressed on the basis of globulin contents, instead of the total protein contents, since reduction of the total plasma protein in infections may be due to the reduction of albumin. To elucidate this, albumin-globulin ratio was studied by electrophoresis on the cellulose acetate membrane. The kininogen contents were divided by the amounts of albumin or globulin obtained from A/G ratio and the total protein concentration in plasma. As it was expected, that kininogens per mg albumin were not significantly different among three groups, because albumin concentrations were reduced with the increased severity of the disease (Fig. 6). The kininogen per mg globulin in the mild cases, however, was also not clearly different from that in the control group. The finding, that the significant decrease of kininogen per ml plasma in the mild cases was a false reduction, coincided well with no detection of kinin. From these results, we may conclude that the kinin system was involved only in the severe group of mice. Reduced amounts of albumin were also reported in mice with the lapse of time after inoculation by Tella and Maegraith (6), although the total serum protein did not decrease in their experiment. The latter finding can be explained by the simultaneous increase of globulin. In the present experiments, the globulin amounts increased only in the severe cases of the infection, whereas those in the mild cases (below 30% in parasitaemia) decreased significantly.

Concerning the determination method of kininogen, which was reported by Diniz and Carvalho (10) and was used in this experiments, recent papers reported the contamination of bradykinin potentiators besides released bradykinin after incubation of acidified, denatured plasma with trypsin (11, 12). Since this was confirmed by us and the improvement of the method has not been established, attention was paid as described in the methods of the present paper.

The consumption of kininogen in the severe state of malaria confirmed qualitatively the findings reported by Tella and Macgraith (1) and Onabanjo and Macgraith (2). According to the authors in monkeys infected by *Plasmodium knowlesi*, kininogen per ml plasma fell to 8% of the preinfection level or was depleted at the final stage, whereas kininogen level of mice in the present experiments did not fall to less than 37% for the lowest value in the severe group. The quantitative difference may be accounted for the preparatory process of plasma as well as experimental conditions including the species difference. Dialysis of the precipitate after 50% saturation by ammonium sulphate, which was used by these researchers (1), may reduce the kininogen contents, because Jacobsen reported that pseudoglobulin contains no substrate I (high molecular weight kininogen) (17).

Onabanjo and Macgraith also presented the paper, showing the increased numbers of the fraction containing kallikrein in serum of the primates infected by malaria after fractionation of plasma (2). Various tests with these fractions such as the vascular permeability and diapedesis of leucocytes in the skin and the brain were also done (18-21). The increase or activation of kallikrein in plasma may be conceivable, and may explain the consumption of kininogen in plasma, but the plasma edetate stable substrate which they used for the substrate of kallikrein may have contained the reduced amount of kininogen I (high molecular weight kininogen), because contact with silicate powder induces the activation of the glass-activated plasma kallikrein and consumes kininogen I.

In the present experiments, the kininase activity in plasma increased markedly in the infected mice. Part of the increased activity may be due to microscopical haemolysis, caused by the malarial infection, as erythrocytes have strong kininase activity. The same observation was reported in the *P. knowlesi*-infected primates (3).

In the infected mice, systemic arterial pressure was significantly lower than that in the control mice, as reported in monkeys (22, 23). The observation may well be a reflection of the pathophysiological role of kinin, which was obviously extracted from blood with consumption of its precursor in plasma. The authors, however, are of the opinion that more definite evidence on the presence of the circulating kinin is necessary for explanation of the hypotension by the extracted kinin.

Intravenous injection of peptide-like kinin potentiators may be a trial to detect the circulating kinin in the blood stream. Kato and Suzuki isolated five bradykinin potentiators from the venom of a snake (*Agkistrodon halys blomhoffii*) (24)(25) and determined the amino acid sequence of three potentiators B, C and E (26-28), which were synthesized by Sakakibara et al. (29). Bradykinin potentiator C has the following amino acid sequence; Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Isole-Pro-Pro. The pharmacological activities of kinin potentiator C are reported as enhancement of the contraction of guinea-pig ileum and the blood pressure fall by bradykinin and the inhibition of kininase and angiotensin-I converting enzyme in the lung (29).

It was expected that on intravenous injection of potentiator C into mice of the severe cases of the infection might produce further continuous hypotension, when kinin is circu-

lating in the blood stream. The results proved contrary. Systemic arterial pressure in the infected mice did not change, as in the non-infected mice, after slow injection of the peptide. However, the interpretation of the result was not so simple, because the intravenous injection of potentiator C failed to potentiate the hypotensive response to bradykinin in the infected mice. The amounts of potentiator C used were 0.3 or 3 $\mu\text{g}/10\text{g}$ body weight and were sufficient to enhance the response to bradykinin 3 to 5 times as large as that before potentiator C. Consequently, even if circulating kinin is present, the intravenous injection of potentiator C could not produce further hypotension in the infected mice. The reason that the response to bradykinin was not enhanced by potentiator C was not clarified in this experiment. It might be speculated that kininase in the lung had already been inactivated, in combination to the low reactivity of the blood vessels to bradykinin in the infected mice, as shown in Table 4. In any case, this may be one of the pathological features of the malarial infection in mice.

The presence of the circulating kinin in blood stream was challenged by another preliminary experiment; the increased permeability of venules. However, intravenous injection of 5% pontamine sky blue or colloidal carbon to infected mice did not reveal any obvious increase of the vascular permeability. Further study is necessary.

The systemic arterial pressure was significantly lower in the infected mice and the hypotension may be partly accounted for kinin extracted. The results of the present investigations however and not prove the active role of the detected kinin on the hypotension in the infected animals.

The involvement of the kinin system was proved in the severe cases with parasitaemia above 30%, only when examined on the day 7 of infection. The results from mice experiments do not indicate however whether the same is true with the lapse of time after infection. Nevertheless, we know that mice with low parasitaemia on day 7 after inoculation increase their own degree of parasitaemia gradually within a week to become fatal. Therefore, the present results could be extrapolated to the course of the infection after inoculation.

The present results did not provide any clue on the mechanism of activation of the kinin system in mouse malaria, however, since globulin fraction did increase in the severe group in the present experiment, the kinin release might be related to antigen-antibody reaction. Further studies are necessary on this respect.

SUMMARY

1. Mice were infected in malaria with *Plasmodium berghei* (NK 65). The degrees of parasitaemia on day 7 after inoculation were distributed from 4 to 72%, having a peak between 30 and 40%.
2. Kininogen contents per ml plasma on day 7 of infection decreased in conjunction with the increased degrees of parasitaemia. The kininogen contents were significantly reduced in the mild cases (below 30% of parasitaemia) as well as in the severe cases (above 30% of parasitaemia).
3. The increase of the degrees of parasitaemia was accompanied with the reduction

of haematocrit, body weight and plasma protein concentration. The kininogen per mg protein was significantly reduced in the severe cases, whereas no reduction was observed in the mild cases.

4. The reduction of kininogen contents per mg globulin was again not significant in the mild cases, in spite of the significant reduction in the severe cases. The kininogen contents per mg albumin were not significantly different among the control, mild and severe groups.

5. Free kinin in venous blood increased in the severe cases, although it did not increase in the mild cases. It can be concluded from these results that the kinin system was involved in the severe cases, whereas the involvement was not clearly proved in the mild cases.

6. Kininase activity increased significantly in the infected mice.

7. Systemic arterial pressure was obviously lower in the infected mice than in the control mice. This finding might suggest that kinin extracted from blood of the infected mice decreased the systemic arterial pressure.

8. Bradykinin potentiator C from a snake venom was used to prove the circulating kinin in the role on blood stream, in connection with the role on low blood pressure of the infected mice. However, the trial was not successful, because, in the infected mice, dotentiator C did not potentiate the hypotensive responses to bradykinin.

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