Studies on Viral-Induced Anemia in Horses Infected with Equine Infectious Anemia Virus

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ABSTRACT. The mechanism of anemia of equine infectious anemia (EIA) was investigated using erythrocytes (RBCs) from five horses experimentally inoculated with the virus. The RBCs collected from these horses in pyrexial and viremic periods were found to have high titers of EIA virus. These RBCs were liable to be lysed in fresh guinea pig serum and to be phagocytized by cultivated horse leukocytes comparing with those collected in the preinoculation and incubation periods. All horses showed decrease in RBC counts followed by the first typical fever after inoculation though some of them did not have detectable antibody by virus neutralization, hemagglutination inhibition and immunodiffusion tests in these periods. Complement activity of horse sera were decreased in some degree after the peak of pyrexia. So it seems to be concluded that the interaction of RBCs with EIA virus, complement attraction on them without antiviral antibody and successive hemolysis and phagocytosis of them play important roles in the induction of anemia in the infected horses. However, complement coated RBCs were hardly detected by immunofluorescent test in the peripheral blood, probably because they were destructed and eliminated continuously from the circulatory system without accumulation.—**KEY WORDS:** equine, erythrocyte, horse, infectious anemia, sideroleukocyte.

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Equine infectious anemia (EIA) is an acute or chronic viral disease of horses and which is primary characterized by intermittent fever with viremia and anemia [4, 7]. The pathogenesis of the anemia has not been completely elucidated. Bone marrow suppression [12, 26] and destruction of erythrocytes (RBCs) [13, 15, 16] are considered as principal causes of anemia. The destruction of RBCs was documented by the decrease of their life span [13,15], and complement attachment on them and successive their osmotic fragility [11] were supposed to be one of the causes of it. However, the mechanism of complement binding to RBCs has not yet been elucidated. On the other hand, since EIA virus possesses a hemagglutinin to horse RBCs [21], it has been suggested that the virus can attach on RBCs in horse body. In the *in vitro* experiment system, it was indicated that hemagglutinin adsorbed RBCs could

attract complement [23] and some of them were lysed or phagocytized by cultivated leukocytes [23, 24]. So, we tried to determine whether these phenomena were indeed occurring in EIA virus infected horses and whether they were working as causes of the anemia. In this report, using RBCs collected from EIA virus-infected horses in progress of disease, virus and complement attachment on RBCs, hemolysis under guinea pig complement and phagocytosis by cultivated horse leukocytes were examined. The relation of these results and the symptoms of infected horses was also discussed.

MATERIALS AND METHODS

Experimental horses and samplings: Five horses were experimentally infected with Wyoming strain of EIA virus [10]. The virus material was lyophilized serum collected from a horse infected with the strain at the

Table 1. Inoculated virus dose and clinical symptoms of experimental horses

Horse No.	Breed	Sex	Inoculation (log TCID ₅₀ /S.C.)	Incubation (days)	Clinical symptom
H-616	Arab	Male	3.75	13	Acute
H-617	Arab	Male	5.75	8	Acute
H-618	Pony	Female	4.75	7	Acute
H-619	Pony	Female	3.75	13	Chronic
H-620	Pony	Female	2.75	21	Chronic

first pyrexia and contained about $10^{5.75}$ TCID₅₀/0.1ml of virus infectivity titer. Details about horses and inoculated virus titers are shown in Table 1. Body temperature was taken twice a day over the experimental period. Hematological examinations including the test for sideroleukocytes were carried out at least once a week when their body temperature was normal and every day during feverish period using the ethylene diamine tetraacetic acid treated blood taken from the jugular vein. Total white blood cells and RBC counts, hemoglobin value, and hematocrite value were determined using Coulter Counter (Model Zf: Coulter electronic Inc., Hialeh, Fla., USA). Sideroleukocytes were observed using the method reported by Ishii *et al.* [6]. Virus neutralization (VN) [10], hemagglutination inhibition (HI) [21] and immunodiffusion (ID) [14] tests were carried out as described previously. The horses which showed chronic symptoms after onset of the disease were inoculated intramuscularly from 62 days after infection with five consecutive daily dose of 0.2mg of dexamethasone (Corson P; Takeda Pharmaceutical Co., Japan) [8] per Kilogram of body weight.

Virus titration and observation of complement coated erythrocytes: Erythrocytes obtained from heparinized jugular vein blood were washed five times each with 40 volumes of phosphate buffered saline solution (PBS). Washed RBCs were suspended at a 10% concentration in Eagle minimum

essential medium (MEM) and lysed by freeze-thawing. Fresh serum was obtained as described in the following section. Infectivity titration of these samples were carried out by the end point method in tube cultures of normal horse leukocytes [9].

Detection of complement on RBCs were carried out by immunofluorescent test using FITC conjugated anti-horse complement rabbit serum as reported previously [23]. Briefly, 1×10^7 of RBCs in heparinized blood were washed twice with PBS and suspended in 0.2 ml of FITC conjugated anti-horse complement rabbit serum for 30 min in ice water. The RBCs were washed again twice with PBS, resuspended in PBS and observed with a fluorescence microscope (Photomicroscope III; Carl Zeiss, West Germany).

Titration of complement activity in experimental horse serum: Blood collected serially from experimental horses were kept at 37°C for 30 min. The sera were separated from the blood clot after centrifugation at $1,000 \times g$ for 30 min at 4°C, and stored at -80°C. All stocked serum samples were tested for the complement activity simultaneously using the same sensitized sheep RBCs with rabbit hemolysin under the same condition. Zero point two ml of diluted serum was mixed with 0.2ml of veronal buffered saline solution containing divalent cations (VBS) in glass tubes (1.3×10cm) and then 0.2 ml of a 1.5% sensitized sheep RBC suspension in VBS was added. After incubation at 37°C for 2 hr, the mixture

were centrifuged and the supernatant was removed. Sedimented cells in each tube were lysed adding 0.6ml of distilled water. The degree of hemolysis was calculated from the hemoglobin value of the sedimented RBCs which determined by extinction measurements at a wave length of 540nm using a digital double beam programmable multi-wavelength spectrophotometer (UVIDEK-1M; Nihon Bunkou Co., Tokyo, Japan). Percent of hemolysis was read from a standard curve drawn from completely lysed RBCs at different concentrations. The reciprocal of the serum dilution showing 50% hemolysis was considered as the complement activity titer.

Phagocytosis of RBCs from infected horses by cultivated leukocytes: Leukocytes obtained from a normal horse were cultivated in glass bottles at 37°C for 24 hr, and adherent cells were collected and suspended at a concentration of $5.0 \times 10^6/\text{ml}$ in an assay medium which consisted of 95% Eagle MEM and 5% inactivated horse serum. The leukocyte suspension in an amount of 0.5ml was added to each well (16mm in diameter) of a 24-well multiwell plate (FB-16-24-TC; Limbro, Hamden, Conn., USA) and cultivated at 37°C in a humidified 5% CO₂-95% air incubator. Erythrocytes which collected from experimental horses and radiolabeled with ⁵¹Cr were added at the concentration of $1 \times 10^7/\text{well}$ to cultivated leukocytes. After incubation at 37°C for 2 hours, free RBCs were lysed and removed. Phagocytized RBCs were calculated from the radioactivity of leukocytes determined in a gamma counter (Beckman gamma 5500 counter system; Beckman Instruments Inc., Fullerton, Calif., USA). Details of leukocytes cultivation, assay medium, radiolabeling of RBCs and procedures of phagocytosis assay used in this study were reported previously [24].

Erythrocyte lysis by complement: Horse RBCs obtained from heparinized jugular vein blood were washed twice with PBS and

adjusted as a 1.5% suspension in PBS. Zero point two ml of the RBC suspension was mixed with 0.4ml of undiluted fresh horse serum or diluted fresh guinea pig serum containing 8 units of complement activity. After placing at 37°C for 90 min, nonlysed cells were sedimented by centrifugation. The degree of hemolysis was determined by the detection of hemoglobin in the supernatant by extinction measurement at a wave range of 540nm.

Variation of hemoglobin levels in plasma: Heparinized blood collected from jugular vein was immediately centrifuged at $1,000 \times g$ for 20 min and the resulting plasma layer was collected. Turbidity in plasma by lipemia was removed by mixing equal volume of ethyl ether for 5 min and centrifugation at $500 \times g$ for 10 min. Variation of hemoglobin levels in the plasma was determined by extinction measurement at a wave length of 540nm.

RESULTS

All the five horses showed typical fever after inoculation. Two horses, H616 and H617 of them, were acute cases and died at the first pyrexia. Antibody to the virus was not detected by VN, HI and ID tests. Another horse, H618, was also an acute case and died after its body temperature rose to about 40°C and continued for two weeks. Antibodies were detected by HI and ID tests 15 days after inoculation but VN antibody was not found for life. The other two horses, H619 and H620, showed chronic symptoms after onset of the disease and the former manifested recurrent fever 48 days after infection, but the later continued to maintain normal body temperature. Both horses were induced typical febrile responses by inoculation intramuscularly with dexamethasone.

Their body temperature, RBC numbers, hemolytic complement activity titer in

serum, phagocytosis of RBCs by cultivated leukocytes, ratio of RBC lysis under 8 units of guinea pig complement, virus infective titers in serum and on RBCs, sideroleukocyte appearance and VN, HI and ID antibody titers were fluctuated accompanied with progress of the disease. As to these five experimental horses, they are compared between the preinoculation periods and the first feverish periods (Table 2). Especially as to the three horses (H617, H618 and H619) of them, their variations accompanied with the lapse of time are illustrated (Figs. 1, 2 and 3).

The virus began to increase in the serum and on RBCs accompanied with the rise in body temperature and was detectable during the first pyrexial period in all infected horses. Infective titers of virus in serum and

on RBCs were more than $10^{6.00}\text{TCID}_{50}/0.1\text{ml}$ and $10^{3.00}\text{TCID}_{50}/0.1\text{ml}$ respectively in two acute cases of horses when their body temperatures were more than 41°C . On the other hand, in the case of H618, whose body temperature rose to about 40°C and continued for two weeks, virus titer on RBCs increased gradually from $10^{1.00}\text{TCID}_{50}/0.1\text{ml}$ on the first feverish day to $10^{4.00}\text{TCID}_{50}/0.1\text{ml}$ on the 14th day, though virus titer in serum was maintained between $10^{3.25}$ and $10^{5.00}\text{TCID}_{50}/0.1\text{ml}$ during the feverish days. In two chronic cases of horses, the virus titers in the serum and on RBCs decreased along with the fall in body temperature and it was often undetectable when body temperature became normal.

Erythrocyte counts were reduced correlated with the increase of body temperature

Table 2. Clinical and immunological responses of five horses experimentally infected with EIA virus in the first feverish period

	Time period	Horse No.				
		616	617	618	619	620
First fever (Days p.i.)		13-20	8-13	7-29	13-17	21-23
Temperature ($^{\circ}\text{C}$)	{ Pre ^{a)}	37.3	37.5	37.9	37.8	37.6
	{ Feverish ^{b)}	41.3	41.6	40.4	41.4	40.2
RBC ($\times 10^6/\text{mm}^3$)	{ Pre ^{a)}	845	818	713	845	756
	{ Feverish ^{c)}	674	666	551	724	741
Antibody Feverish	{ ID ^{d)}	—	—	—~+ ^{g)}	+	+
	{ HI ^{e)}	—	—	—~+ ^{g)}	—	+
	{ VN ^{f)}	—	—	—	—	—
Hemolytic complement	{ Pre ^{a)}	9.14	8.80	9.14	12.80	8.00
	{ Feverish ^{c)}	6.20	6.20	4.00	8.00	8.00
RBC lysis (%)	{ Pre ^{a)}	52.6	12.8	10.4	8.8	13.0
	{ Feverish ^{b)}	85.0	22.4	18.0	20.2	11.8
RBC Phagocytosis	{ Pre ^{a)}	1.43	1.25	1.16	1.16	1.28
	{ Feverish ^{b)}	1.93	1.91	1.88	2.11	1.75

a) Average of 4 days before infection.

b) The highest in the feverish period.

c) The lowest in the feverish period.

d) Immunodiffusion.

e) Hemagglutinin inhibition.

f) Virus neutralization.

g) Detected from 15 days after infection.

Fig. 1. Clinical and immunological responses of Horse 617 (acute case). 1) Ratio of phagocytosis of infected horse erythrocytes and control horse erythrocytes (Relative phagocytic ratio). 2) Infectivity of erythrocytes (open bar) and serum (closed bar) shown as histogram in terms of log TCID₅₀/0.1ml. 3) Reciprocal of serum dilution induced 50% hemolysis of sensitized sheep erythrocytes. 4) Percentage of hemolysis of erythrocytes under 8 units of guinea pig complement. 5) Number of sideroleukocytes found among about 10,000 leukocytes. —: zero, +: 1, ++: 2–5, +++: 6–20, ++++: >21. 6) Immunodiffusion antibody; —: negative, +: curved faint line, ++: line appeared, +++: clear line appeared. Antibody to EIA virus was not detected by hemagglutination inhibition and virus neutralization tests for life.

in all infected horses. However, as far as the observation by immunofluorescein test with anti-horse complement rabbit serum, few of RBCs were coated with complement in the blood collected from jugular vein during the pyrexia. The ratio of positive RBCs was at most 0.1% including those of which surface

Fig. 2. Clinical and immunological responses of horse 618 (acute case). See footnotes 1)–6) of Fig. 1. Antibody to EIA virus was detected from 15 days after infection by hemagglutination inhibition test but not detected by virus neutralization test for life.

were stained partially or sectionally, even when the body temperature increased to more than 41°C.

Sideroleukocytes appeared in the blood from jugular vein of the four experimentally infected horses approximately from the second day of continued pyrexial days and increased in numbers, and had been detectable until several days after the fall in body temperature. However, in the case of H620, only a small numbers of sideroleukocytes had been detected even just after the peak of pyrexia.

Since the phagocytizing activity of the leukocytes from a healthy horse which cultivated for 24 hr was changeable because of unknown factors of cultivation, it was difficult to prepare a steady condition of leukocyte culture for phagocytosis assay. Ratio of phagocytized RBCs from infected horses was less than 2% and these from a control healthy horse fluctuated between 0.25 and 0.82%. Therefore, numbers of

H620, which also showed a chronic form, variation of complement activity titer was not clear and the relationship with the symptoms of the disease was not evident. It was observed that sera of every experimental horses turned untransparent during and after the period of pyrexia probably because of lipemia.

Variation of hemoglobin levels was not observed in the plasma collected serially from the five experimentally infected horses before, during and after the pyrexia.

DISCUSSION

All the five horses showed the typical symptoms of EIA. Three horses were acute forms and two horses were chronic forms. All of them showed decrease in RBC count followed by typical fever after inoculation with EIA virus. Some horses showed increase in RBC counts after the stage of fever crisis probably due to concentration of RBCs because of dehydration caused by high body temperature.

Since the decline of iron transfer from bone marrow to RBCs [17, 18] and the atrophy of the hematopoietic tissue were observed [12, 26] in the febrile stage in EIA virus infected horses, the hypofunction of hematopoiesis in bone marrow and such organs is supposed to be one of the causes of anemia. On the other hand, since complement attachment on RBCs and resultant decrease of RBC life span [11], appearance of auto-antibodies against RBCs [19], and intravascular hemolysis [13] in and around the febrile stage were also reported, intravascular destruction of RBCs has been supposed to be important in the occurrence of the anemia. Present experiments were focused on the interaction between virus hemagglutinin and RBCs and consequential their reactions. Essentially, almost the same results were obtained from these experimentally EIA virus infected horses by

several tests which were done to elucidate a mechanism of anemia. So, anemia of these horses was supposed to be caused by the identical or resemble mechanisms, though each horse showed different clinical symptoms.

Both hemolytic ratio and relative phagocytic ratio rose accompanied with the rise of body temperature, viremia and attachment of viruses on RBCs. These results suggest that several reactions which followed interaction of intact virus or hemagglutinin subunits with RBCs works as one of the causes of anemia. However, appearance of the complement attached RBCs and both hemolysis and phagocytosis of RBCs in this study were not so obvious as those observed in our previous *in vitro* experiments [23, 24]. In those *in vitro* experiments, RBCs were treated with high concentration of virus. On the other hand, in the infected horse body, virus propagation, virus and complement attachment on RBCs, their lysis and phagocytosis by leukocytes were probably occurring continuously. These RBCs would be under the clearance control and be eliminated from the circulating blood without accumulation. Accumulation of a large quantity of sideroleukocytes which seemed to be eliminated from the peripheral blood had been observed in spleen [5, 25].

It was reported that auto-antibodies against RBCs appear and bind to RBCs in horses infected with EIA virus [19]. In the present experiments, erythrocyte sedimentation in the heparinized jugular vein blood was increased during the pyrexia in some horses, and such a phenomenon suggested that the RBCs might be agglutinated with some immunoglobulin. However, as far as the observation by direct immunofluorescein test with anti-horse immunoglobulin rabbit serum (E. Y. Lab. INC., San Mateo, Calif., USA), the RBCs with fluorescence were hardly detected in the blood even in the feverish periods.

Studies on the auto-antibody and the role of the auto-antibody reaction in the cause of anemia still remain for future investigations.

It was reported that intravascular hemolysis occurs in horses exposed to EIA virus [13] and our previous study also indicated that such hemolysis is likely [23]. However, results of the present study demonstrated that such a hemolysis was not obvious and seem to play minor role as a cause of anemia. On the other hand, indirect hemolysis mediated by phagocytes would be efficient. Our previous study demonstrated that the phagocytosis by macrophage was induced by small quantity of hemagglutinin on RBCs [24]. During the study, it was also observed that contaminated neutrocytes ingested affected RBCs. Neutrocytes are known to have receptors against immunoglobulin and third complement component [1, 3], rich in phagocytizing activity and account for almost half of circulating leukocytes. A large amount of sideroleukocytes which originated from macrophages and neutrocytes, had been found in spleen [5, 25, 26]. So, their role in the phagocytosis of affected RBCs would be also significant.

Decrease of RBC numbers and appearance of sideroleukocytes were observed in and after the pyrexial period accompanied with viremia though there were no detectable antibodies by CF, HI and VN tests. Hemagglutinin adsorbed RBCs could activate complement without antiserum in the *in vitro* experiment [23]. These phenomena suggested that these antiviral antibodies were not necessarily required in the mechanism of anemia. However, in present study, many sideroleukocytes were found in peripheral blood of a horse which manifested recurrent fever and had a high titer of HI antibody about in two months after infection. In our previous report, EIA virus was coexisted with HI antibody in the virus-infected horse [22]. Virus-antibody mixture which had lost HA activity could

induce hemolysis and phagocytosis of RBCs though antibody addition did not obviously enhance the reactions [23, 24]. So, it is supposable that virus specific immune complexes on RBCs could also attract complement. The hemolysis by complement activation seems to be induced by virus specific immune complexes on RBCs, as well as by virus mediated RBCs.

Decreased complement activity was observed in serum samples taken in the later part or at the end of active clinical disease. The phenomenon was already reported by Perryman *et al.* [20] using single radial immunodiffusion test. The consumption of circulating blood C3 might have been more serious than it looked in horses infected with EIA virus, since decreased complement level would be generally replenished rapidly in the system [2].

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要 約

馬伝染性貧血ウイルス感染馬におけるウイルス性貧血：泉 対 博・甲野雄次（家畜衛生試験場研究第2部）
 ——馬伝染性貧血ウイルスを接種した5頭の馬についてウイルスと赤血球の結合、変性赤血球への補体の結合・活性化、その結果生ずる溶血またはマクロファージ等による捕食について調べた。発熱、ウイルス血症を示したときの赤血球には、ウイルスが付着しており、モルモット補体存在下で37°Cで接種前または潜伏期赤血球と比べて溶血しやすく、健康馬由来培養マクロファージによって捕食されやすかった。すべての例で初回発熱の後期及び下熱直後に赤血球数が減少したが、その時点では一部の例でゲル内沈降試験、HI試験、中和試験によって抗体が検出できなかった。発熱とともに血清補体活性価は低下した。