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AFRICAN SWINE FEVER. II. FUNCTIONAL DISTURBANCES OF THROMBOCYTES IN PIGS INFECTED WITH VIRULENT HAEMADSORBING AND NON-HAEMADSORBING VIRUS ISOLATES

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ABSTRACT

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Increased bleeding time, impaired blood clot retraction and decreased thrombocyte aggregation were observed in pigs infected with virulent haemadsorbing and non-haemadsorbing African swine fever virus isolates. These changes appeared to be more frequent and more severe in pigs infected with the haemadsorbing virus isolates than in those infected with the non-haemadsorbing isolate. Moreover, the onset and severity of these changes followed the numerical decrease and morphological damage to these cells apparent in the last 2–3 days of the disease.

INTRODUCTION

Acute African swine fever (ASF) is a lethal, highly infectious viral disease of domestic pigs, often characterized by widespread haemorrhage (De Kock, Robinson & Keppel, 1940; Maurer, Griesemer & Jones, 1958; Moulton & Coggins, 1968; Edwards, 1983).

Haemorrhage in acute ASF may follow vascular damage as a result of replication of the virus in endothelial cells (Wilkinson & Wardley, 1978), thrombocytopenia, defective fibrin clot formation or a combination of these (Edwards, 1983; Edwards, Dodds & Slauson, 1985). Thrombocytopenia occurred terminally only in pigs infected with ASF virus isolates which caused death within 8 days (Edwards, 1983; Edwards *et al.*, 1985). They postulated thrombocytopenia to be immune-mediated, while no evidence for decreased thrombocytopoiesis could be found (Edwards, 1983; Edwards & Dodds, 1985; Edwards *et al.*, 1985). Marked morphological changes, including cytoplasmic swelling, vacuolation, fragmentation and loss of dense granules became apparent in thrombocytes of pigs infected with virulent haemadsorbing (HD) and non-haemadsorbing (NHD)-ASF virus isolates during the last 2 days of life. Replicating and mature ASF viral particles were demonstrated by transmission electron microscopy in a small percentage of damaged blood platelets in these pigs (Neser, Phillips, Thomson, Gainaru & Coetzee, 1986).

Thrombocytes fulfil essential functions during haemostasis by forming haemostatic platelet plugs which serve to arrest haemorrhage (Karpatkin & Holmsen, 1983; Zucker, 1983). These functions represent complex responses to subendothelial collagen, thrombin, adenosine diphosphate (ADP) and metabolic products of arachidonic acid. On stimulation, thrombocytes which normally circulate as flattened discs, will contract and secrete their internal granule contents, develop pseudopodia, aggregate together and adhere to endothelium. Thrombocytes having characteristics similar to those of myocytes, contraction is by submembranous actinmyosin filaments. A marginal bundle of microtubules helps to maintain the discoid shape of platelets and promotes secretion after stimulation. The internal granule contents secreted after contraction include ADP, adenosine triphosphate (ATP), serotonin, catecholamines, platelet factor 4 and fibrinogen. Release of these factors is an energy dependent process and does not follow disruption of organelles. Platelet factor 4 appears to be a potent activator for thrombocyte aggregation and blood clotting. Fibrinogen binds to specific receptors on thrombocyte pseudopodia and promotes clot retraction, thereby trapping other blood cells within the clot.

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Prolonged bleeding time, decreased thrombocyte aggregation and impaired clot retraction are strong indications of thrombocyte dysfunction (Hougie, 1983; Weiss, 1983; Pitney & Brozovic, 1984).

The present investigation was an attempt to evaluate the functional effects of the reduced thrombocyte counts and the morphological changes previously described in thrombocytes of pigs infected with virulent HD- and NHD-ASF virus isolates (Neser *et al.*, 1986). An attempt was also made to study possible differences in the degree of thrombocyte dysfunction in pigs infected with virulent HD- and NHD-ASF virus isolates.

MATERIALS AND METHODS

Twenty-five cross-bred pigs 4–6 months old were housed and infected with 4 virulent HD-ASF virus isolates [Control (CV), 951, Malawi (MWI) and Dominican Republic (DR)] and a virulent NHD-ASF virus isolate (Lillie-148), was described in a previous report (Neser *et al.*, 1986).

Bleeding time: A modification of Duke's method (Schalm, Jain & Carroll, 1975) was used to determine the bleeding time in 5 pigs (Pigs 1, 2, 11–13) ifected with a HD-ASF virus isolate (Table 1a) and in 5 pigs (Pigs 17, 20, 23–25) infected with a NHD-ASF isolate (Table 1b). Briefly, pigs were placed in lateral recumbency under light anaesthesia as described by Neser *et al.* (1986) to prevent ear twitching. The hair on the anterior apical ear margin was clipped and the skin wiped as cleanly as possible with dry tissue paper. Small wedge-shaped incisions *c.* 5 mm deep and *c.* 20 mm apart were made in the clipped area daily or every 2nd day from 2–4 days before infection up until death (Tables 1a & b). Blood drops were picked-up at 30–60 s intervals onto the edge of a circular filter paper sheet. Bleeding time was measured from the time of incision to the cessation of haemorrhage.

Blood clot reraction: A modification of the methods described by Schalm *et al.* (1975) and Hougie (1983) were applied to measure blood clot retraction in all 13 pigs infected with the HD-ASF virus isolates (Table 2a) and in 10 pigs (Pigs 15, 16, 18–25) inoculated with the NHD-ASF virus isolate (Table 2b). Ten millilitres of venous blood was collected in sterile glass tubes free of anticoagulant (Vacutainer)[1] from the *anterior* vena cava (Neser *et al.*, 1986). A wire paper clip was bent open and twisted around to point in opposite directions. Immediately after the tube was filled to the 10 mℓ mark, the clip was suspended into the blood with the upper hook over the edge of the tube and the lower hook directed

[1] Vacutainer, Raydem Laboratories, P.O. Box 391606, Bramley, 2018, RSA

TABLE 1 a Bleeding time before and after infection with the control HD-ASF virus isolate

Pig No.	Days before and after virus inoculation. Bleeding time (min)									Survival time (days)
	−3	−2	−1	0	1	2	3	4	5	
1	—	—	—	4	—	—	17	—	†	4
2	—	—	—	5	—	—	4	25	†	4
11	6	—	—	—	5	—	—	12	17	5
12	5	—	—	—	5	—	—	5	30	5
13	10	—	—	—	5	—	—	7	30	5

TABLE 1 b Bleeding time before and after infection with the Lillie NHD-ASF virus isolate

Pig No.	Days before and after virus inoculation. Bleeding time (min)											Survival time (days)
	−2	−1	0	1	2	3	4	5	6	7	8	
17	10	—	—	—	—	5	—	—	3	—	—	8
20	10	—	10	—	6	—	—	7	—	5	†	7
23	—	—	7	—	5	—	5	—	—	6	35	8
24	—	—	7	—	5	—	5	—	—	10	15	8
25	—	—	20	—	6	—	5	—	—	4	5	8

— = Not determined
† = Animal dead

towards the centre of the lumen near the bottom of the tube. Blood was allowed to clot for *c.* 5 min after which the clot was carefully separated from the glass tube wall. Tubes with blood were incubated for 5–7 h at 37 °C while clot retraction and serum expression were checked every few hours. The changes in the relative amounts of dark red and greyish-white fibrinous elements of blood clots were subjectively assessed visually according to the following semiquantitative scale.

Dark red blood elements: 100 %=4B; 75 %=3B; 50 %=2B; 25 %=B

Greyish-white fibrinous blood elements: 100 %=4F; 75 %=3F; 50 %=2F; 25 %=F

Clots were subsequently carefully lifted by the upper hook and removed from the serum, where possible, and observed for changes in colour, composition and integrity. The serum was allowed to stand until the upper serum volume could be distinguished visually from the lower sediment fraction. Where possible, serum volumes were measured by carefully pipetting them as separate fractions to a calibrated 10 mℓ glass measuring cylinder. Sediment fraction volumes could not be measured accurately in all instances because of its gelatinous consistency and could therefore not be considered quantitatively in the assessment of clot retraction.

Thrombocyte aggregation: Thrombocyte aggregation was studied on certain days before and after Pigs 1–10 had been infected with HD-ASF virus isolates (Table 3a) and Pigs 17–22 with the NHD-ASF virus isolate (Table 3b). Blood specimens were drawn from the vena cava anterior (Neser *et al.*, 1986) and preserved in a 3,8 % sodium citrate solution (9 parts of blood to 1 part of anticoagulant).

Aggregation studies were performed in a Payton[2] dual channel aggregometer, applying a modification of Blakely's method (Payton Associates Limited)[2]. Adenosine diphosphate (ADP), diluted ADP (1:10) and collagen

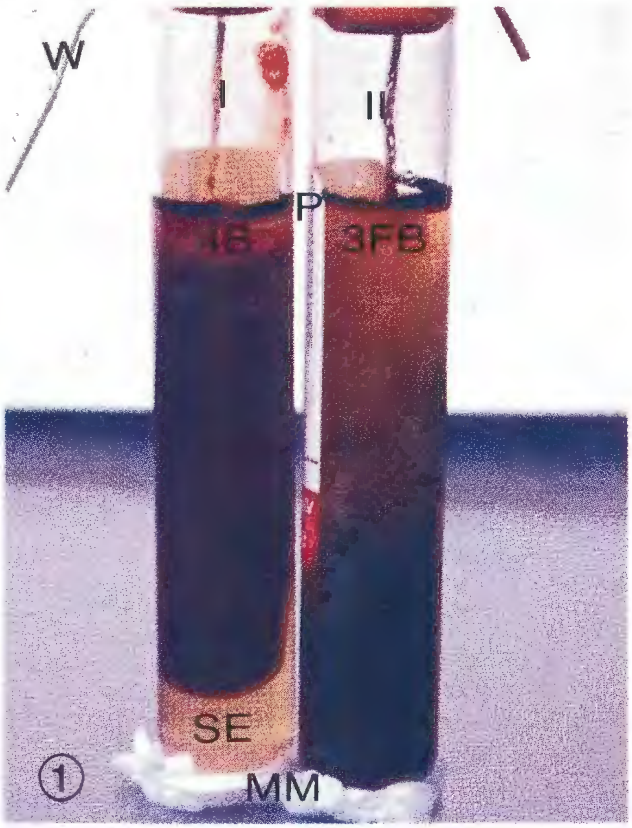

FIG. 1 Typical procine blood clot retraction before infection (I) with firmly contracted homogenous dark red blood clot (4B) and clear serum (S) expressed; impaired blood clot retraction post-infection (II) with poorly contracted greyish-white fibrinous blood clot (3FB) with no clear serum expressed; glass tubes marked at 10 mℓ volume with black pen line (P) and fixed in upright position with mounting material (MM). W = wire paper clip bent to form hook

aggregation reagents were used (Cluster, 1980)[3]. To prepare thrombocyte-rich plasma (TRP), citrated blood specimens were centrifuged at 200 *g* for 10 min, after which

[2] Payton Associates Ltd., 85-9 Nantucket Blvd., Scarborough, Ontario, Canada

[3] Cluster American Dade, Division of American Hospital Supply Corporation, Miami, FL 33152, USA

TABLE 2 a Sequential blood clot retraction (evaluated by volume of serum expressed and appearance of the retracted blood clot) before and after infection with 4 different HD-ASF virus isolates

Pig No.	Virus inoculated	Blood clot retraction	Days before and after virus inoculation									Survival time (days)
			−3	−2	−1	0	1	2	3	4	5	
1	CV	V	—	—	—	6	—	—	4	—	†	4
		AP	—	—	—	4B	—	—	4B	—		
2	CV	V	—	—	—	—	—	—	4	2	†	4
		AP	—	—	—	4B	—	—	4B	4B		
3	CV	V	6,5	—	—	6,0	—	—	4,0	—	—	5
		AP	4B	—	—	4B	—	—	F3B	—	—	
4	CV	V	6,5	—	—	6,4	—	—	5,2	2,0	—	5
		AP	4B	—	—	4B	—	—	4B	3FB	—	
5	951	V	6,0	—	—	6,8	—	—	6,2	0	†	4
		AP	4B	—	—	4B	—	—	4B	3FB		
6	951	V	—	—	—	7,0	—	—	5,0	0	†	4
		AP	—	—	—	4B	—	—	4B	4B		
7	MWI	V	6,5	—	—	6,2	—	—	5,0	0	†	4
		AP	4B	—	—	4B	—	—	4B	3FB		
8	MWI	V	6,0	—	—	6,2	—	—	6,0	0	1,0	5
		AP	4B	—	—	4B	—	—	4B	4B	4B	
9	DR	V	5,5	—	—	6,2	—	—	4,0	4,5	—	5
		AP	4B	—	—	4B	—	—	2B2F	4B	—	
10	DR	V	6,6	—	—	5,9	—	—	6,6	4,8	0,0	5
		AP	4B	—	—	4B	—	—	4B	3BF	3BF	
11	CV	V	5,0	—	—	—	4,6	—	—	0	0	5
		AP	4B	—	—	—	4B	—	—	4B	4B	
12	CV	V	5,0	—	—	—	3,3	—	—	1,0	0	5
		AP	4B	—	—	—	4B	—	—	4B	2B2F	
13	CV	V	4,6	—	—	—	4,0	—	—	0	0	5
		AP	4B	—	—	—	4B	—	—	4B	3BF	
Total 13									Mean ±	SD	4,6 ± 0,5	

V = Volume of serum measured in millilitres
B = Dark red blood elements
F = Greyish-white fibrinous blood elements
All other conventions as for previous tables

AP = Subjective assessment of blood clot appearance
1 = 25 %. 2 = 50 %, 3 = 75 % and 4 = 100 %

the TRP was drawn off and sotred at room temperature. The remaining citrated blood specimens were re-centrifuged at 750 *g* for 30 min to prepare thrombocyte-poor plasma (TPP). Platelet rich plasma suspensions were diluted to platelet counts ranging from 200–500 × $10^9/\ell$, wherever possible using TPP specimens according to Blakely's method (Payton Associates Limited)[2]. Platelet aggregation was measured over 5 min for each specimen at 37 °C at a stirring speed of 900 RPM, while the recorder chart speed was set at 50 mm/min.

The definition of aggregation parameters

(i) Maximal aggregation (M) was measured on the chart curve in milimetres (Fig. 2a) as the greatest absolute vertical deflection of the aggregation curve due to maximal light transmission (Weiss, 1983; Pitney & Brozovic, 1984).

(ii) The maximal rate of aggregation (S) was also measured for all 3 aggregation reagents (Fig. 2a). This parameter was determined by calculating the slope of a line drawn tangentially to the initial rapid wave of the aggregaton curve (Weiss, 1983; Pitney & Brozovic, 1984).

(iii) The "lag time" (L) was measured in the case of aggregation with collagen as the distance in mm from the time of addition of the agent to the first vertical deflection of the aggregation curve (Fig. 2a) (Skokoza, Zucker, Jerushalmy & Grant, 1967; Cluster, 1980; Pitney & Brozovic, 1984).

Statistical analysis

The survival times of the pigs from both groups were compared statistically and tested for significance (at P = 0,000015), using the adjusted Welch-aspin-t-test value

TABLE 2b Sequential blood clot retraction (evaluated by volume of serum expressed and appearance of the retracted blood clot) before and after infection with the Lillie-148 (NHD)-ASF virus isolate

Pig No.	Blood clot retraction	Days before and after virus inoculation																						Survival time (days)
		−7	−6	−5	−4	−3	−2	−1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
15	V								6,0	—	—	—	—	—	—	5,0	—	2,5	—	3,8	—	†			12
	AP								4B	—	—	—	—	—	—	4B	—	4B	—	4B	—				
16	V								5,0	—	7,0	—	6,0	—	—	†									6
	AP								4B	—	4B	—	3BF	—	—	—									
18	V	5,0	—	7,0	—	—	6,0	—	6,0	—	6,5	—	—	6,0	—	6,2	—	6,0	—	—	5,5	—	5,0	†	14
	AP	4B	—	4B	—	—	4B	—	4B	—	4B	—	—	4B	—	4B	—	4B	—	—	4B	—	—		
19	V	6,0	—	8,0	—	—	6,5	—	6,4	—	6,0	—	—	4,7	—	5,0	—	4,0	—	—	†				11
	AP	4B	—	4B	—	—	4B	—	4B	—	4B	—	—	4B	—	4B	—	4B	—	—					
20	V						6,0	—	5,0	—	5,5	—	—	4,0	—	2,0	†								7
	AP						4B	—	4B	—	4B	—	—	4B	—	4B									
21	V						6,1	—	6,2	—	—	—	5,5	—	—	—	5,0	5,0	—	†					10
	AP						4B	—	4B	—	—	—	4B	—	—	—	3BF	3BF	—						
22	V						6,4	—	6,5	—	—	—	2,0	—	—	6,0	6,0	5,0	—	†					10
	AP						4B	—	4B	—	—	—	2B2F	—	—	4B	3BF	2B2F	—						
23	V								5,5	—	5,6	—	6,0	—	—	0	0	†							8
	AP								4B	—	4B	—	4B	—	—	4B	4B								
24	V								5,5	—	4,5	—	5,0	—	—	0	0	†							8
	AP								4B	—	4B	—	4B	—	—	B3F	B3F								
25	V								5,5	—	4,5	—	5,8	—	—	0	0	†							8
	AP								4B	—	4B	—	4B	—	—	B3F	B3F								
Total 10																								Mean ± SD = 9,4 ± 2,46	

FIG. 2a–d Matelet-aggregation curves displayed with different reagents before and after infection with virulent HD- and NHD-ASF virus isolated

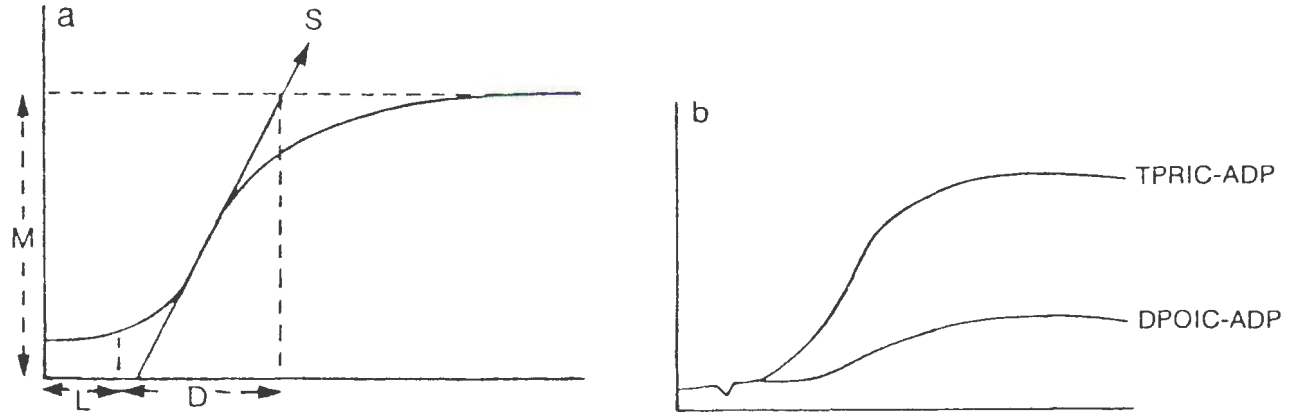

FIG. 2 (a) Maximal platelet aggregation (M) measured in millimetres (mm), maximal rate of aggregation (S) calculated as the initial slope of the aggregation curve, horizontal distance in mm (D) from the tangential line intersection to the vertical intersection on the abscissa, used to calculate S and the horizontal distance in mm (L = lag time in s) from the time of addition of the collagen aggregation reagent to the first vertical deflection on the chart curve

FIG. 2 (b) Typical pre-infection porcine platelet aggregation curve with concentrated ADP (TPRIC-ADP) and a depressed post-infection porcine aggregation curve with concentrated ADP (DPOIC-ADP) using Cluster[3] platelet aggregation reagents

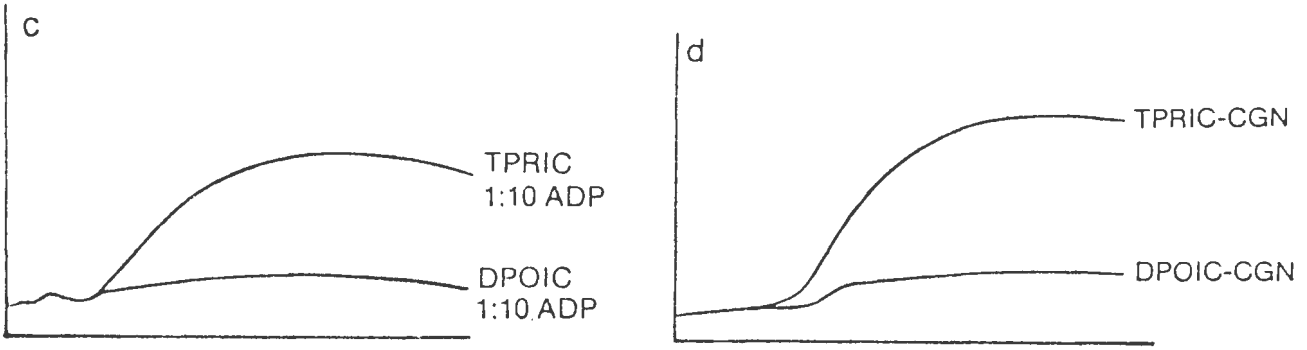

FIG. 2 (c) Typical pre-infection procine platelet aggregation curve with diluted 1:10 ADP (TPRIC) and a depressed post-infection porcine aggregation curve with 1:10 ADP (DPOIC) using Cluster[3] platelet aggregation reagents

FIG. 2 (d) Typical pre-infection porcine platelet aggregation curve with collagen (TPRIC-CGN) and a depressed post-infection porcine aggregation curve with collagen (DPOIC-CGN) using Cluster[3] platelet aggregation reagents

(Cressie & Whitford, 1986). The pre- and post-inoculation bleeding time values were also compared and the differences tested for significance by the χ^2 test (at 1 degree of freedom).

Results

Bleeding time: Sequential bleeding times for pigs from both groups are recorded in Tables 1a & b except where determinations were not done because of unexpected deaths. The bleeding time was markedly prolonged on the day of death in 4 out of 5 pigs (Pigs 2, 11–13) and in 1 pig (Pig 1) on the day before death, after infection with the HD-ASF virus isolate (Table 1a). On the contrary, in pigs infected with the NHD-ASF virus isolate, the bleeding time was noticeably prolonged only on the day of death in 1 of the 5 pigs (Pig 23). The bleeding time was also prolonged in 1 pig (Pig 25) on the day of infection (Table 1b). There was thus a prolongation of the bleeding time terminally in a greater number of pigs infected with the HD-ASF isolate (Table 1a) than in pigs infected with the NHD-ASF virus isolate (Table 1b).

Blood clot retraction: Sequential blood clot retraction values for pigs from both groups were recorded to the last 2 days before death (Tables 2a & b). The wire hooks incorporated into the blood clots did not appear to impede clot retraction, since clots regularly retracted to 50 % or more of the original blood volume in the uninfected pigs (Tables 2a & b). The volume of serum expressed was markedly reduced during the last 2 days of life in all 9 of the pigs (Pigs 2, 5–8, 10–13) infected with the HD-ASF isolates (Table 2a) and in 4 of the 5 pigs (Pigs 18, 20, 23–25) infected with the NHD-ASF virus isolate where determinations were possible on the last day of life (Table 2b). The most noticeable changes in blood clot composition, colour and integrity were characterized by a separation of the homogeneous red blood clot masses into loosely coherent mixtures of greyish-white fibrinous and dark red blood elements (Fig. 1), indicated by the symbols B & F (Tables 2a & b). These changes were most conspicuous in 6 of the 13 pigs (Pigs 3, 4, 5, 7, 9 & 12) infected with the HD-ASF virus isolates (Table 2a), and in 5 of the 10 pigs (Pigs 16, 21, 22, 24 & 25) infected with the NHD-ASF virus isolate (Table 2b). Depending on the degree of clot disintegration, the sediment fraction volumes varied from 0 to *c.* 1 mℓ before infection to almost 10 mℓ in some terminally infected specimens. Precise measurements were not possible.

Blood platelet aggregation: In uninfected pigs thrombocytes produced strong aggregation responses comparable to those of normal pigs (Bowie, Owen, Zollman, Thompson & Fass, 1973) with the reagents used in this study. Aggregation with concentrated ADP produced a strong monophasic curve, a slightly weaker reversible curve with diluted ADP (1:10) and a typical lag phase curve with collagen (Fig. 2b-d) (Weiss, 1983; Zucker, 1983).

After infection, however, there was a reduction of the aggregation response where determinable during the last 2 days of life which became particularly noticeable on the day of death in 8 of the 10 pigs (Pigs 2–6, 8–10) infected with the HD-ASF isolates (Table 3a). On the

TABLE 3 (a) Aggregation response of blood platelets (lag time, initial slope and maximal height of curve) to ADP, diluted ADP and collagen, measured before and after infection with 4 different HD-ASF virus isolates

Pig No.	Virus inoculated	Aggregating agent	Days before and after virus inoculation																			Survival time (days)
			−3			−2			−1			0			1	2	3			4			5			
			L	S	M	L	S	M	L	S	M	L	S	M	L S M	L S M	L	S	M	L	S	M	L	S	M	
1	CV	ADP	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0,36	8,0		—	—	—				4
		ADP 1:10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0,29	6,0		—	—	—		†		
		CCN	—	—	—	—	—	—	—	—	—	—	—	—	—	—	60	0,22	3,0	—	—	—				
2	CV	ADP	—	—	—	—	—	—	—	—	—	0,60	56		—	—	0,66	30		0	3					4
		ADP 1:10	—	—	—	—	—	—	—	—	—	0,50	30		—	—	0,60	22		0,30	8			†		
		CCN	—	—	—	—	—	—	—	—	—	—	—	—	—	—	64	0,31	8	0	3					
3	CV	ADP	—			0,76	52		—	—	—	—	—	—	—	—	0,40	18		0	5		—	—	—	5
		ADP 1:10	—			0,55	28		—	—	—	—	—	—	—	—	0	6		0	2		—	—	—	
		CCN	—			110	0,31	34	—	—	—	—	—	—	—	—	68	0,28	28	0	2		—	—	—	
4	CV	ADP	—			0,71	36		—	—	—	—	—	—	—	—	0,75	53		0	2		—	—	—	5
		ADP 1:10	—			0,48	26		—	—	—	—	—	—	—	—	—	—	—	0	3		—	—	—	
		CCN	—			110	0,20	18	—	—	—	—	—	—	—	—	130	0,63	48	0	3		—	—	—	
5	951	ADP	—			0,70	56		—	—	—	—	—	—	—	—	0,16	14		0	2					4
		ADP 1:10	—			0,46	28		—	—	—	—	—	—	—	—	0,20	8		0	3			†		
		CCN	—			58	0,71	50	—	—	—	—	—	—	—	—	45	0,26	26	0	2					
6	951	ADP	—			0,58	44		—	—	—	—	—	—	—	—	0,50	30		0,1	4					4
		ADP 1:10	—			0,30	14		—	—	—	—	—	—	—	—	—	—	—	0	4			†		
		CCN	—			73	0,50	46	—	—	—	—	—	—	—	—	120	0,46	39	0	6					
7	MN	ADP	—	—	—	—	—	—	—		0,90	56		—	—	—	0,47	35		0,33	24					4
		ADP 1:10	—	—	—	—	—	—	—		0,50	42		—	—	—	0,20	13		0,15	10			†		
		CCN	—	—	—	—	—	—	—	70	0,70	54		—	—	—	100	0,40	31	70	0,33	34				
8	MN	ADP	—	—	—	—	—	—	—		0,80	50		—	—	—	0,36	0,30		0	3			0	4	5
		ADP 1:10	—	—	—	—	—	—	—		0,60	44		—	—	—	0,35	0,14		0	6			0	7	
		CCN	—	—	—	—	—	—	—	70	0,65	52		—	—	—	120	0,20	25	300	0	4			0	5	
9	DR	ADP	—	—	—	—	—	—	—		0,80	48		—	—	—	1,00	48		0,25	10		—	—	—	5
		ADP 1:10	—	—	—	—	—	—	—		0,50	24		—	—	—	0,36	20		0,33	6		—	—	—	
		CCN	—	—	—	—	—	—	—	50	0,70	55		—	—	—	68	0,66	53	66	0,25	12		—	—	—	
10	DR	ADP	—	—	—	—	—	—	—		0,80	54		—	—	—	0,6	45		0,25	12			0	4	5
		ADP 1:10	—	—	—	—	—	—	—		0,60	36		—	—	—	0,25	20		0,33	7			0	7	
		CCN	—	—	—	—	—	—	—	65	0,60	57		—	—	—	85	0,48	49	80	0,25	20		100	0	5	

CV = Control virus (HD)-ASF virus isolate
MW = Malawi virus (HD)-ASF virus isolate
451 = (HD)-ASF virus isolate
DR = Dominican Republic (HD)-ASF virus isolate

ADP = Adenosine diphosphate
ADP1:10 = Diluted ADP
CCN = Collagen

L = Lag phase (millimetres)
M = Maximal aggregation (millimetres)
All other conventions as for previous tables

TABLE 3(b) Aggregation response of blood platelets (lag time, initial slope and maximal height of aggregation curve) to ADP, diluted ADP and collagen measured before and after infection with the NHD-ASF virus isolate (Lillie-148)

Pig No.	Aggr-gation agent	Days before and after virus inoculation																																		Survival (days)
		−7		−6		−5		−4		−3		−2		−1		0		+1		+2		+3		4		5		6		7		8		9		10		11		12		13		14		
		LSM		LSM		LSM		LSM		LSM		L	S	M	LSM	L	S	M	LSM	L	S	M	LSM	L	S	M	L	S	M	L	S	M	L	S	M	LSM	L	S	M	LSM	L	S	M	
17	ADP	---		---		---		---		---		-	-	-	---	-	-	-	---	-	-	-	---	-	-	-	-	1,30	51	-	-	-	-	-	-	†									8
	ADP 1:10	---		---		---		---		---		-	-	-	---	-	-	-	---	-	-	-	---	-	-	-	-	0,90	32	-	-	-	-	-	-									
	CGN	---		---		---		---		---		-	-	-	---	-	-	-	---	-	-	-	---	-	-	-	18	-	19	-	-	-	-	-	-									
18	ADP	---		---		---		---		---		-	1	54	---	-	0,6	41	---	53	0,75	40	---	-	-	-	69	0,66	46	-	-	-	59	0,88	52	-	-	-	-	0,70	43	-	-	-	14
	ADP 1:10	---		---		---		---		---		-	0,60	31	---	-	0,50	62	---	-	0,60	30	---	-	-	-	-	0,50	16	-	-	-	-	0,50	30	-	-	-	-	0,40	22	-	-	-	
	CGN	---		---		---		---		---		83	0,80	56	---	80	0,71	39	---	55	0,57	0,46	---	-	-	-	83	0,40	38	-	-	-	52	0,83	64	-	-	-	56	0,50	35	-	-	-	
19	ADP	---		---		---		---		---		-	0,60	46	---	-	0,50	38	---	-	0,65	44	---	-	-	-	-	0,55	42	-	-	-	72	0,66	48	-	-	-	-	0,80	44	-	-	-	11
	ADP 1:10	---		---		---		---		---		-	0,40	20	---	-	0,40	13	---	-	0,37	22	---	-	-	-	-	0,30	14	-	-	-	-	0,50	28	-	-	-	-	0,40	28	-	-	-	
	CGN	---		---		---		---		---			0,61	53	---	90	0,59	58	---	65	0,50	46	---	-	-	-	94	0,40	42	-	-	-	-	0,66	58	-	-	-	102	0,30	24	-	-	-	
20	ADP	---		---		---		---		---		-	1,4	44	---	-	1,7	55	---	-	0,75	33	---	-	-	-	-	0,30	13	-	-	-	-	0,30	6										7
	ADP 1:10	---		---		---		---		---		-	0,80	30	---	-	1,2	65	---	-	0,71	10	---	-	-	-	-	0,45	18	-	-	-	-	0,30	10	†									
	CGN	---		---		---		---		---		6	1,45	17	---	65	0,70	58	---	3	1,00	6	---	-	-	-	-	0,22	4	-	-	-	-	0,3	8										
21	ADP	---		---		---		---		---		-	-	-	---	-	-	-	---	-	-	-	---	-	0,75	52	-	-	-	-	0,50	44	-	0,37	38	-	-	-	-	0,5	25	-	-	-	10
	ADP 1:10	---		---		---		---		---		-	-	-	---	-	-	-	---	-	-	-	---	-	0,80	44	-	-	-	-	0,38	30	-	0,36	26	-	-	-	-	0,37	24	-	-	-	
	CGN	---		---		---		---		---		-	-	-	---	-	-	-	---	-	-	-	---	60	0,70	64	-	-	-	85	0,43	54	110	0,38	30	-	-	-	-	0,10	20	-	-	-	
22	ADP	---		---		---		---		---		-	-	-	---	-	-	-	---	-	-	-	---	-	0,70	45	-	-	-	-	0,64	54	-	0,50	38	-	-	-	-	0,40	25	-	-	-	10
	ADP 1:10	---		---		---		---		---		-	-	-	---	-	-	-	---	-	-	-	---	-	0,50	24	-	-	-	-	0,10	62	90	0,50	38	-	-	-	-	0,25	12	-	-	-	
	CGN	---		---		---		---		---		-	-	-	---	-	-	-	---	-	-	-	---	80	1,37	42	-	-	-	-	0,64	62	90	0,50	48	-	-	-	-	0,25	30	-	-	-	

ADP = Adenosine diphosphate
ADP 1:10 = Diluted adenosine diphosphate (1:10)
CGN = Collagen
All conventions as for previous Tables

contrary, a reduction of the aggregation response became noticeable only in one pig (Pig 20) of the 4 animals (Pigs 18, 20–22) infected with the NHD-ASF isolate, where determinations were done during the last 2 days of life (Table 3b).

The maximal extent of thrombocyte aggregation indicated by the degree of vertical deflection on the chart curve (Weiss, 1983; Pitney & Brozovic, 1984) showed a marked reduction with all 3 aggregation reagents (Fig. 2b-d) in all affected specimens (Tables 3a & b). Although the rate of thrombocyte aggregation represented by the initial slope of the aggregation curve (Weiss, 1983; Pitney & Brozovic, 1984) was variable, it was also markedly reduced with all 3 aggregation reagents (Fig. 2b-d) in the majority of affected specimens infected with the HD-ASF isolates (Tables 3a & b). The lag phase, characteristic for the aggregation response with collagen (Weiss, 1983; Zucker, 1983; Pitney & Brozovic, 1984) was markedly delayed and often indeterminable, owing to the low slope of the aggregation curve (Fig. 2b-d) in the majority of affected specimens infected with the HD-ASF virus isolates (Tables 3a & b).

As the disease progressed, it became difficult, and impossible in some instances, to obtain the desired minimal thrombocyte count of 200 × $10^9/\ell$ (Payton Associates Limited[2]) in the terminal specimens, because of a progressive reduction of thrombocyte numbers. This was particularly noticeable in the pigs infected with the HD-ASF virus isolates. However, notwithstanding the inadequate thrombocyte counts in several thrombocyte plasma specimens, a discrete, although weak, aggregation response could be measured in the terminal specimens.

Survival time: The difference in survival times between the 2 groups of pigs was obviously signigicant when compared with the t-test (P = 0,000015) using the adjusted Welch-Aspin-t-value (Cressie & Whitford, 1986) (Table 1).

Discussion

Daily titrations of ASF virus in blood were not performed in the present study. However, viraemic levels at death were determined in a limited number of pigs. These were high in both groups (Neser *et al.*, 1986) and were comparable with maximal levels reached 72–96 h post inoculation in other studies (Plowright, Parker & Staple, 1968; Wardley & Wilkinson, 1977a; Edwards, 1983).

The bleeding time was measured only in a limited number of pigs infected with the HD- and NHD-ASF virus isolates, and the values were noticeably longer (15–35 min) than those reported in normal Yorkshire-Hampshire strain pigs (1,25–4 min) (Bowie *et al.*, 1973). The saline immersion method used by these authors differs markedly from Duke's earcut-method (Schalm *et al.*, 1975; Hougie, 1983) used in our study. There was a significant (χ^2>5 %, 1 degree of freedom) prolongation of the bleeding time during the last 2 days of life in 5 pigs infected with the HD-ASF virus isolate and in 1 of the pigs infected with the NHD-ASF virus isolate, the onset of which corresponds to the terminal appearance of thrombocytopenia (Edwards, 1983; Edwards *et al.*, 1985b) and morphological changes in the platelets in acute ASF (Neser *et al.*, 1986).

The single increased bleeding time on the day of infection in 1 pig infected with the NHD-ASF virus isolate was difficult to explain. It is suspected that the traumatization of the ears by inadvertent handling of the pig by its ears could have played a role. Holding of pigs by the ears should be avoided and ears must be kept static while measuring bleeding time. Results obtained by means of

Duke's earcut-method should be interpreted with caution. Prolonged bleeding time but normal ADP induced thrombocyte aggregation was also reported in swine with Von Willebrand's disease (Bowie *et al.*, 1973). Von Willebrand's factor or plasma factor VIII:vWF (FVIII: vWF) is required for the adhesion of thrombocytes to blood vessel walls in the formation of a normal haemostatic platelet plug (Hovig & Stormorken, 1974; Weiss, 1974; Zucker, 1983). Plasma FVIII:vWF levels, ristocetin induced platelet aggregation, platelet retention in glass bead columns and platelet adhesion to subendothelium could not be determined in this study to establish a possible FVIII:vWF deficiency (Salzman, 1963; Tschopp, Weiss & Baumgartner, 1974; Sakariassen, Bolhuis & Sixma, 1979; Zucker, 1983).

There is some evidence for increased prostaglandin E_2 (PGE_2) secretion by macrophages and endothelial cells in acute ASF (Anderson, 1986). It was suggested that an elevated level of PGE_2 may act as a potent vasodilator and inhibitor of platelet aggregation in the disease (Smith, 1980; Anderson, 1986). A scarcity of platelet thrombi in various tissues was observed microscopically in pigs infected with the HD- and NHD-ASF virus isolates in our study (Neser, unpublished data, 1987) as well as by Edwards (1983). Although these findings may be partly due to thrombocytopenia (Neser *et al.*, 1986), the impaired platelet aggregation demonstrated *in vitro* in this study should be considered as being at least partly responsible for the deficient aggregation of platelets and the few platelet thrombi seen in tissues.

Clot retraction is not a purely quantitative test and should be interpreted as normal, equivocal or defective based on a normal retraction of *c.* 50 % in man. Nevertheless, much useful information may be obtained by inspecting the appearance of the blood clot (Hougie, 1983). In the determination of clot retraction, the wire hooks suspended into the tubes provided a valuable method of separating the retracted blood clots from the expressed serum with minimal disturbance. Judged by the volumes of serum expressed (50 % except in 1 specimen) and the firm clots formed, clot retraction was normal before infection (Hougie, 1983; Zucker, 1983). Marked defective blood clot retraction occurred abruptly terminally in the disease. A greater number of determinations at shorter intervals during the last 2–3 days before death will be required to follow the course of impaired clot retraction more closely. However, it should be taken into account that the vena cava anterior can only sustain a limited number of venipunctures, especially in pigs suffering from thrombocytopenia and platelet dysfunction. The function of thrombocytes in a blood clot formed *in vitro* corresponds to a certain extent to their function in a physiologic platelet plug. Reduced clot retraction may therefore reflect thrombocytopenia or impaired thrombocyte function in the formation of a haemostatic platelet plug (De Clerk, Borgers, De Gaetano & Vermylen, 1975; Zucker, 1983).

Increased fibrinogen and fibrin degradation product (FDP) levels and prolonged thrombin clotting times were measured in plasma specimens of pigs infected with these virulent HD and NHD-ASF isolates (Neser & Coetzee, 1984, prepublished results). Furthermore, partial disintegration or dissolution of blood clots as observed in this study was considered evidence of intravascular coagulation or a hyperfibrinolytic state (Hougie, 1983), with excessive FDP's formed in plasma. These products may interfere with platelet adhesion, aggregation and release reactions, and lead to disturbed blood coagulation through their competitive inhibition of the clotting action of thrombin on fibrinogen (Slauson & Cooper, 1982; Francis & Marder 1983).

Decreased thrombocyte aggregation also occurred terminally and somewhat abruptly in pigs infected with both virulent HD- and NHD-ASF virus isolates, but it was more frequent in pigs infected with the HD-ASF virus isolates. Further studies at shorter intervals during the course of the disease will be required to add reliability to our findings. Diminished platelet aggregation in pigs may be the result of insufficient platelets in the test caused by thrombocytopenia (Neser *et al.*, 1986). Although thrombocyte counts dropped to below normal in most of the pigs they were only markedly decreased in a few animals (Neser *et al.*, 1986).

The increased bleeding time, decreased clot retraction and impaired platelet aggregation in the majority of pigs infected with the HD-ASF isolates in our study would suggest some influence of the haemadsorption factor (HD) on the functional disturbances of thrombocytes. However, impaired clot retraction appeared to be more frequent and severe in pigs infected with the NHD-ASF virus which died within less than 9 days (mean survival period for group 9,4) than in those that lived for longer. From these results it would seem that clot retraction depends on the course of the disease and the virulence of the isolate rather than on the HD-characteristic of the ASF virus isolate. Unfortunately bleeding time, clot retraction and platelet aggregation could only be determined in a limited number of pigs infected with the NHD-ASF isolate on the last day of life due to unpredictable deaths. Firm conclusions could therefore not be reached on the *in vivo* effects of the HD-characteristics or virulence of the ASF-virus isolates on the disturbances of thrombocyte function.

Lie (1968) showed that in piglets with iso-immune thrombocytopenia, haemorrhage only occurred when platelet counts fell below $40 \times 10^9/\ell$. Decreased thrombocyte aggregation, impaired clot retraction and prolonged bleeding times have been reported in congenital thrombocyte membrane and dense granule defects (Weiss, 1983). Morphological changes such as cytoplasmic vacuolation, fragmentation and loss of dense granules in thrombocytes of ASF infected pigs (Neser *et al.*, 1986), may therefore in addition to thrombocytopenia and the presence of FDP's have a bearing on the functional derangements and haemorrhage seen in animals suffering from ASF.

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CORRIGENDUM

Page 154, left column, second paragraph, second sentence should read:

"It was suggested that an elevated level of PGE_2 may act as a potent vasodilator and *promotor* of platelet aggregation in the disease (Smith 1980; Anderson 1986)."