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In vitro inactivation of complement by a serum factor present in Junin-virus infected guinea-pigs

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Summary. A serum factor (s) of guinea-pigs infected with Junin virus, the etiological agent of Argentine haemorrhagic fever, is endowed with a potent anti-complementary activity. It is resistant to heat (56°, 30 min) and elutes from a Sephadex G-200 column between albumin and haemoglobin. It is ineffective in the presence of EDTA or EGTA and does not sediment at 82,000 ***g***. It has no direct effect on C4 unless functional C1 is present. However, it induces C1 activation that consumes C4 haemolytic activity in normal human and guinea-pig sera. The evidence presented in this report demonstrates that the complement activation observed in experimental Argentine haemorrhagic fever is at least in part due to a direct effect of this serum factor on the classical complement pathway.

INTRODUCTION

Junin-virus infected guinea-pigs provide a suitable model for the study of the pathogenesis of Argentine haemorrhagic fever (AHF). Complement (C) alterations coincident with activation of the coagulation system have been described in the acute stage of the experimental disease (Budzko & Acobettro, 1973; Molinas, Paz, Rimoldi & de Bracco, 1978).

There is substantial evidence that activation of one of the acute phase reactants (coagulation, C, kinins, and fibrinolysis) may trigger the other inflammatory responses (Kaplan, 1974). C activation has been demonstrated in a variety of systemic infections characterized with disorders of haemostasis (Bokisch, Top, Russell, Dixon & Müller-Eberhard, 1973; Tomar & Kolchins, 1972; Arguello, de Bracco & Sanchez Avalos, 1973). In another viral haemorrhagic fever, dengue, immune complexes are thought to produce massive activation of both the C and the clotting systems (Bokisch, 1974; Bokisch *et al.*, 1973). A pathogenic mechanism of this kind could be proposed in experimental AHF. However, abnormalities of the C profile, resembling the C pattern observed when the classical pathway is activated (Fig. 1) (Budzko, Rimoldi, Acobettro & de Bracco, 1975) occur in the absence of detectable anti-viral antibodies in experimental AHF (Guerrero, Boxaca, Weissenbacher & Frigerio, 1977). Moreover, the presence of active proteolytic enzymes (Kierszenbaum, Budzko & Parodi, 1970), cationic proteins (Venge & Olsson, 1975) or a direct effect of virus on the C system (Cooper, Welsh & Oldstone, 1976) could produce similar results (Budzko *et al.*, 1975).

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The results of this study demonstrate that a factor present in the serum of infected guinea-pigs (AHF gpS) activates the classical pathway in normal human (NHS) and guinea-pig serum (NgpS). Furthermore, activation by this factor resembles the activation produced by leucocyte extracts (PLE).

MATERIALS AND METHODS

Sources of Junin-virus infected guinea-pig serum (AHF gpS)

Random bred guinea-pigs, weighing 250–350 g were infected with 100 DL50 of XJ strain of Junin virus (Kierszenbaum *et al.*, 1970; Budzko *et al.*, 1975). AHF gpS was obtained by heart puncture on the 9th, 11th and 13th day post-infection; a pool of infected sera was stored at −20°. Complement (Budzko *et al.*, 1975) and coagulation studies (Molinas *et al.*, 1978) have been carried out previously with the same serum samples.

Sources of normal guinea-pig serum (NgpS) and human serum (NHS)

NgpS and NHS were obtained from blood which was allowed to clot at room temperature for 1 h. The sera were divided into aliquots and stored at −70°.

Polymorphonuclear leucocyte extracts (PLE)

PLE were obtained from dextran-sedimented guinea-pig leucocytes according to the method of Plow & Edgington (1975).

Buffers and other reagents

Heated NHS (56°, 30 min) and C1r-deficient serum (de Bracco, Moncada, Windhorst & Stroud, 1974) were used as sources of C4 in C1-deficient sera. Isotonic NaCl solution buffered to pH 7·3–7·4 with veronal was supplemented with 0·1% gelatin, $1{\cdot}0 \times 10^{-3}$ M Mg and $1{\cdot}5 \times 10^{-4}$ M Ca (VBS). Low ionic strength isotonic buffer (DGVBS) was obtained by adjusting the resistance of VBS to 0·065 RSC with 5% dextrose. Divalent cation chelating solutions were prepared with VBS containing 0·01 M ethylenediaminotetraacetic acid (EDTA) or 1,2-bis(2-dicarboxymethylaminoethoxy) ethane (EGTA).

Complement consumption

The ability of AHFgpS and PLE to consume C was determined in reaction mixtures containing equal volumes of the diluted test reagents and 1/2 diluted NHS or 1/10 diluted NgpS. Control reactions, using dilutions of NgpS instead of AHFgpS were set up simultaneously.

The reaction mixtures were incubated for 1 h at 37 or 0°. Residual C activity was measured by the method of Kent & Fife (1963). Residual C4 haemolytic titre was assayed as described by Ruddy & Austen (1967) AHFgpS and NHS were reacted in the presence of 0·01 M EDTA or EGTA in order to assess the requirement of divalent cations in the inactivation reaction. Residual C or C4 activity were tested after reconstitution with Ca and Mg.

Chromatography

AHFgpS (0·5 ml) was chromatographed on a Sephadex G-200 column (Pharmacia, Uppsala, Sweden) measuring 2·5 × 65 cm. Borate buffer (0·25 M, pH 8) was used as eluent. Fractions were tested for C consumption, protein (Lowry, Rosebrough, Farr & Randall, 1951) and IgG concentration (Mancini, Carbonara & Heremans, 1965).

Ultracentrifugation

AHFgpS was diluted 1/20 in VBS and centrifuged at 82,000 *g* for 1 h in a L2 Spinco ultracentrifuge with a SW 40 rotor.

RESULTS

The C-inactivating effect of pooled sera from Junin-virus infected guinea-pigs were tested upon normal human or guinea-pig serum. Table 1 shows that AHFgpS contains a heat-resistant factor (s) which inactivates total C activity and C4 in a dose-dependent way. In the same dilutions, NgpS had no C consuming effect on NHS. On the other hand, when the mixtures were incubated at 0° (Table 2) or in the presence of 0·01 M EDTA or EGTA (Table 1), no C consumption was observed. The results presented in Table 2 demonstrate that the inactivation reaction is more effective in the xenogeneic combination. C inactivation of NgpS by AHFgpS varied from 25 to 10% whereas the consumption of C haemolytic activity of NHS by AHFgpS was always around 50%. Therefore NHS was selected as test reagent.

To determine if the requirement of divalent cations was related to the presence of functional C1 in the test serum (NHS), experiments were done reacting AHFgpS with C1-deficient sera. Either serum from C1r-deficient patients or NHS rendered deficient in C1

Table 1. Decrease of C and C4 haemolytic activity of NHS treated with AHFgpS

Experiment	NHS incubated* at 37°, 1h, with	CH50 % control	C4 % control
Dose response	AHFgpS 1/20	56	37
	1/40	60	65
	1/80	68	123
	NgpS 1/20	126	76
	1/40	115	88
Resistance to heat	AHFgpS 1/20	55	37
	(56°, 30 min) 1/20	52	41
	NgpS (56°, 30 min) 1/20	100	65
Requirement of divalent cations	AHFgpS 1/20, $Ca^{2+}Mg^{2+}$	59	37
	EGTA	116	104
	EDTA	122	92

* NHS was incubated for 1 h at 37° with different dilutions of AHFgpS, NgpS, heated AHFgpS or heated NgpS in the presence or absence of Ca^{2+} and Mg^{2+}. Residual C activity (CH50) or C4 activity (SFU) were expressed as percentage of the titre of NHS incubated with VBS alone (% control).

haemolytic activity by heat treatment were used. The results summarized in Table 3 demonstrate that no C4 was consumed in the absence of active C1.

Complement consumption by guinea-pig PLE

Previous reports (Kierszenbaum *et al.*, 1970) have demonstrated that enzymes of lysosomal origin appeared in the sera of Junin-virus infected guinea-pigs concomitant with the decrease of the leucocyte count. This occurs before the complement and coagulation alterations observed by us (Molinas *et al.*, 1978). In view of the potential C-consuming activity of these substances, the following experiment was carried out. The C-inactivating activity of PLE from normal guinea-pigs was assayed using NHS and NgpS as test sera. PLE were reacted as AHFgpS in the presence or absence of metal ion chelators. The results shown in Table 4 indicate that PLE inactivate C in a dose-dependent way. As was described above with AHFgpS, PLE require divalent cations and react more efficiently in the xenogeneic combination (Table 4).

Table 2. Effect of the temperature of the reaction on the total haemolytic activity of NHS and NgpS treated with AHFgpS or NgpS

	Percentage of control haemolytic activity			
	NHS		NgpS	
Reaction	0°C	37°C	0°C	37°C
AHFgpS	105	55	99	93
NgpS	109	101	135	130

Diluted NHS (1/2) and NgpS (1/10) were reacted with an equal volume of 1/20 AHFgpS, 1/20 NgpS or VBS at 0° or at 37° for 1 h. Residual C haemolytic activity was measured and the results are expressed as the percentage of the haemolytic titre of VBS incubated samples.

Table 3. C4 consumption by AHFgpS requires functional C1

Reaction	C4 (SFU/ml) NHS	Heated NHS	Clr def HS
AHFgpS	1060	2710	35,000
VBS	5150	2470	33,000

C4 haemolytic titre (SFU/ml) of normal human serum (NHS), NHS heated at 56° for 30 min (heated NHS) and C1r-deficient human serum (C1r def HS) was measured after 1 h incubation at 37° with AHFgpS or VBS.

Complement inactivation by AHFgpS is not due to high molecular weight aggregates

In order to establish if high molecular weight aggregates were the heat-stable agents that caused C-inactivation of normal sera two approaches were followed: AHFgpS was filtered through Sephadex G-200 and the C-inactivating factor was surveyed in relation to the elution volume of IgG, albumin and haemoglobin (Fig. 2). Active fractions eluted after the albumin peak, just ahead of haemoglobin. IgG was eluted far away from the peak of inhibitory activity.

In addition, AHFgpS was ultracentrifuged at 82,000 *g* for 1 h. The results of the titrations of the residual C activity of sera treated with the supernatant and bottom fractions are shown in Table 5. Together with the results depicted in Fig. 2, this experiment indicates that the C-inactivating factor present in AHFgpS is not a high molecular weight aggregate.

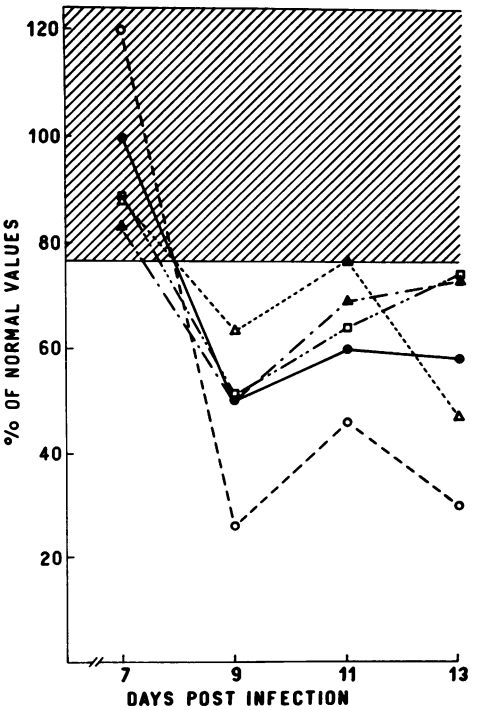

Figure 1. Complement profile of guinea-pigs experimentally infected with 100 DL50 of Junin virus. o, C4; ▵ C1q; ●, CH50; ▲, C2; □ C3.

DISCUSSION

An experimental disease reminiscent of AHF can be induced in guinea-pigs by infection with Junin virus, a member of the arenavirus group (Coto, 1974). In this

Table 4. Effect of the guinea-pig PLE on the haemolytic activity of NHS and NgpS

Experiment	Incubation at 37° 1 h, with	NHS % CH50 control	NgpS % CH50 control
Dose response	PLE 1/2	23	73
	1/4	28	82
	1/8	43	94
	1/16	79	97
Requirement of divalent cations	PLE 1/8, Ca Mg	49	ND
	PLE 1/8, EGTA	92	ND
	PLE 1/8, EDTA	85	ND

NHS or NgpS were incubated at 37° for 1 h with different dilutions of PLE in the presence or absence of Ca and Mg. Residual C activity (CH50) was expressed as percentage of the titre of NHS or NgpS incubated with buffer alone (% control).

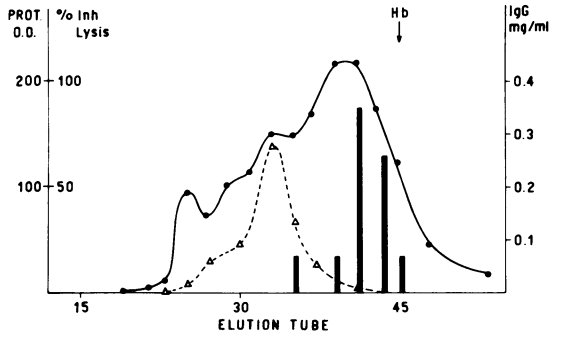

Figure 2. Sephadex G-200 elution pattern of AHFgpS. AHFgpS (0·5 ml) was chromatographed on a Sephadex G-200 column (2·5 × 65 cm). IgG, albumin and haemoglobin present in the serum were used as markers.

model, activation of the complement system has been consistently observed (Budzko & Acobettro, 1973). Activation of the C in Junin-virus infected guinea-pigs proceeds through the classical pathway and the significance of this alteration has been discussed previously (Budzko *et al.*, 1975). Briefly, Junin-virus infected guinea-pigs had reduced C4 and C2 haemolytic activity and decreased C1q and C3 assayed immunochemically (Fig. 1). Activation of the C system was evident 9 days after infection and persisted until death (Budzko *et al.*, 1975). Interestingly, C4 haemolytic activity was more affected than its protein concentration and serum C4 had altered electrophoretic mobility (Rimoldi & de Bracco, 1976). Reduced haemolytic efficiency of C4 has been also observed in human AHF during the acute stage (de Bracco, Rimoldi, Cossio, Rabinovich, Maiztegui, Carballal & Arana, 1978).

In an attempt to explain the alterations of the C system observed in experimental AHF, we searched for factors in infected sera that could induce *in vitro* similar changes in NHS or NgpS. As shown in Tables 1 and 2 AHFgpS induced such alterations in a temperature-dependent reaction. The results listed in Table 1 indicate that the bulk of the C-consuming activity of AHFgpS is thermostable. On the other hand, the presence of divalent cations is necessary for C consumption by AHFgpS. In addition, as demonstrated in Table 3, C1r-deficient and heated sera does not support C4 consumption by AHFgpS. Thus, the presence of functional C1 is a requirement for C4 inactivation by AHFgpS. These results and the fact that the reaction does not occur in the presence of EGTA (Table 1), strongly suggest that the main effect of AHFgpS on the C system is exerted through the classical pathway.

Table 5. Anti-complementary activity of AHFgpS after ultracentrifugation

CH50 (u/ml) of NHS reacted with			
VBS	AHFgpS	AHFgpS UC bottom	AHFgpS UC supernatant
115	68	115	66

C haemolytic activity (CH50) of NHS was measured after reaction at 37° for 1 h with VBS, AHFgpS, bottom (UC bottom) and supernatant (UC supernatant) fractions of AHFgpS ultracentrifuged at 82,000 ***g*** for 1 h.

Certain substances from peripheral leucocytes that consume C activity in a Ca^{2+}-dependent reaction (Table 4) also resist 56° for 30 min. If the granulocytopenia observed in experimental AHF (Gonzalez & Mejszenkier, 1962) was due to peripheral destruction, levels of leucocyte-derived enzymes as those used for the experiments shown in Table 4 could be expected during the terminal phase of the disease. In fact, it is noteworthy that maximum lysosomal enzyme activity was found *in vivo* after 12 days of infection (Kierszenbaum *et al.*, 1970) and sera obtained in this period exhibited the highest *in vitro* C consuming activity and the lowest C4 haemolytic efficiency (Rimoldi & de Bracco, 1976).

Heat-stable, anti-complementary activity may be ascribed to the presence of immune complexes or IgG aggregates. However, in studies performed on kidneys of guinea-pigs infected with Junin virus (Cossio, Laguens, Rabinovich, Carballal, Cabeza-Meckert, Maiztegui, Vasquez & Arana, 1977) no *in vivo* bound globulins were detected in glomerular structures. Furthermore, guinea-pigs died without detectable anti-viral specific antibodies (Guerrero *et al.*, 1977). Nonetheless, in order to investigate if the anti-complementary factor in AHFgpS was related to high molecular weight aggregates, infected sera were filtered through a Sephadex G-200 column and ultracentrifuged at 82,000 ***g***. The results shown in Fig. 2 indicate that the inhibitory activity corresponds to a substance with molecular weight intermediate between albumin and haemoglobin. In accordance with these results, data shown in Table 5 demonstrate that the inhibitory activity remains in the supernatant after ultracentrifugation. These results rule out the possibility that immune complexes are the C-inactivating factors present in AHFgpS.

Massive C activation might play a relevant role in

the pathogeny of experimental AHF. It is associated with activation of the coagulation system, although it is preceded by thrombocytopenia (Molinas *et al.*, 1978). Since immune complexes do not seem to be the factors that trigger C consumption in Junin-virus experimental infection, it can be speculated that massive breakage of granulocytes (Gonzalez & Mejszenkier, 1962) caused by direct cytopathic effect of Junin virus and release of intra-cellular enzymes may in turn provoke the activation of the coagulation and complement systems. The resultant haemostatic and vascular disorders would finally lead to death of the animals.

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