Influence of the terminal complement-complex on reperfusion injury, no-reflow and arrhythmias: a comparison between C6-competent and C6-deficient rabbits

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Abstract

Objective: The complement system has been suggested to play a role in reperfusion injury which may result from an enhanced destruction of myocardial tissue or from an impairment of reflow. We investigated the influence of the C5b-9 complement complex on infarct size, reflow and arrhythmogenesis.

Methods: Twenty-eight C6-competent rabbits and 18 rabbits with congenital C6 deficiency were subjected to either 30 min or 2 h of coronary artery occlusion followed by reperfusion. C6 deficiency was confirmed by the complement titration test and immunohistology. The triphenyl tetrazolium chloride method was used to delineate infarct size. Reflow into infarcted areas was evaluated histologically after an in vivo injection of propidium iodide which served as an early fluorescence microscopic marker of damaged myocardium subjected to reflow. Continuous ECG monitoring allowed the recording of arrhythmias.

Results: After 30 min of coronary artery occlusion infarct size was significantly smaller in C6-deficient rabbits (5.0 ± 2% of the risk region) as compared to C6-competent rabbits (28.4 ± 8.5%, P = 0.0371). The extent of reflow into damaged myocardium was nearly the same in both animal groups at this time (38 ± 9 vs. 39 ± 7% of the risk region). After 2 h of coronary artery occlusion, infarct size was not different between both animal groups, but the extent of reflow into damaged myocardium was significantly smaller in C6-competent rabbits than in C6-deficient rabbits (25 ± 4 vs. 40 ± 4%; P = 0.0185). Two of the 18 C6-deficient rabbits had ventricular arrhythmias (Lown II–IV), none of which was fatal. Eleven of the 28 C6-competent animals had major ventricular arrhythmias which were fatal in 6 rabbits.

Conclusions: These results suggest that the lytic C5b-9 complement complex leads to reperfusion injury in the early phase (30 min) of ischaemia, resulting in a larger infarct. After 2 h of ischaemia, complement activation enhances the no-reflow phenomenon but does not affect infarct size. Finally, the C6 status seems to influence the susceptibility to ventricular arrhythmias after coronary artery occlusion, independent of reperfusion.

Keywords: Complement system; Myocardial infarction; Reperfusion; Myocardial infarct size; Arrhythmias; Rabbit, anesthetized

1. Introduction

The role of the complement system in the pathogenesis of myocardial ischaemic tissue damage has been investigated in several studies [1–7]. We recently observed that terminal C5b-9 complement accumulates rather late in myocardial infarction without reperfusion but rather early if the ischaemic myocardium is reperfused [8]. Therefore C5b-9 complement is more likely to contribute to reperfusion injury than to ischaemic tissue damage in the non-reperfused heart. This contention is in line with other studies which suggest that complement activation plays an important role in myocardial reperfusion injury [9–11]. Smith et
coronary occlusion reduces myocardial infarct size after reperfusion in rats [9]. Soluble complement receptor type 1 suppresses formation of both C5a and lytic C5b-9 complement complex [12]. Whereas C5a is a strong chemotactic and activating factor for neutrophils, C5b-9 complexes generated in cell membranes are cytotoxic [13,14]. The question whether C5a or C5b-9 alone or in conjunction contributed to reperfusion injury remained unanswered in the study of Smith et al. [9]. Homeister et al., however, were able to demonstrate in an isolated heart preparation that complement activation depressed myocardial function and increased coronary perfusion pressure [15]. These effects were not mediated by the anaphylatoxins but were dependent on C8 complement, suggesting that C5b-9 was responsible for these changes [15]. Neutrophils, the main target of the anaphylatoxins, however, were not present in the perfusate and the hearts were not subjected to classical ischemia and reperfusion [15]. For these reasons the question whether C5b-9 complement alone contributes significantly to reperfusion injury was not conclusively answered in the study of Homeister et al. In the present study the influence of C5b-9 on reperfusion injury was therefore studied here in an in vivo model of myocardial infarction and reperfusion.

We first intended to determine histologically the precise localization of C5b-9 in relation to myocardial tissue damage and reflow after shorter and longer periods of coronary artery occlusion followed by reperfusion. Infarct size of congenitally C6-deficient rabbits which were not capable of forming C5b-9 was then compared with that of normal C6-competent rabbits.

C5b-9 complement may influence infarct size after reperfusion either directly by increased destruction of myocardial tissue or indirectly by impairing reflow. The increase in coronary perfusion pressure by complement activation observed by Homeister et al. indirectly suggested that the impairment of reflow might be an important mechanism [15]. We therefore also studied the areas of reflow. The same group was able to demonstrate that myocardial tissue accumulated sodium and calcium and lost potassium as a result of complement activation [15]. This was not surprising since lytic complement affects cell membrane permeability. An attempt was therefore made to demonstrate whether C5b-9 complement complex influences arrhythmogenesis after myocardial infarction and reperfusion.

2. Methods

The present study was performed with permission of the Amt für Gesundheit und Veterinärmedizin of the Freie und Hansestadt Hamburg (No. F 1 56/90). It conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.1. Experimental protocol

Twenty-eight C6-competent and 18 congenitally C6-deficient rabbits of the same race were included in the study. C6-deficient rabbits were originally a gift of Drs. K. and U. Rother (Institute for Immunology, University of Heidelberg, Germany). A breeding colony was established at the Institute of Medical Microbiology and Hygiene, University of Mainz. The animals used in the experiments were usually pairs of homozygotes and heterozygotes from the same litter. C6-deficiency arises from a single gene defect and is not associated with other abnormalities. C6 competence or deficiency remained unknown during the experiment. The determination of infarct size and area of reflow were also performed without knowledge of the C6 status. The latter was determined from blood samples by the complement titration test described below. The rabbits were randomly assigned to 30 min or 2 h of coronary artery occlusion followed by 1 or 3 h of reperfusion.

The animals were sedated by an intramuscular injection of ketamine HCl (4–8 mg per kilogram bodyweight), xylazine (8–9 mg per kilogram body weight) and received atropine (0.1 mg per kilogram bodyweight). Anaesthesia was induced by a continuous intravenous infusion of pentobarbital (12 mg per kilogram bodyweight per hour). Electrodes were placed on the extremities to allow continuous ECG monitoring throughout the procedure. All ventricular ectopies were recorded and classified according to the grading system of Lown [16]. The animals then underwent tracheotomy and were artificially ventilated with a constant tidal volume of 25 ml, a respiratory rate of 50/min and an oxygen flow of 1 l/min ensuring maximal oxygenation in all animals studied. A median thoracotomy was performed and the heart was suspended in a pericardial cradle before a large marginal branch of the circumflex coronary artery was ligated. This coronary artery is a large reproducible vessel in rabbits, which have no left anterior descending coronary artery. The ligature (4.0 prolene) was tefl-arme to prevent artery damage and was maintained using a tourniquet and a clamp.

Reperfusion was allowed to occur by releasing the clamp and retrieving the tourniquet. Fifteen minutes before the end of the reperfusion period propidium iodide (2 mg in 2 ml NaCl 0.9%; Sigma Chemicals GmbH) was injected into the left atrium to permit detection of the area of reflow. The dye entered the damaged cells and led to intense fluorescent staining of the nuclei. At the end of the reperfusion period the ligature was reinstituted and 3 ml of a 1% solution of patent blue violet (Sigma Chemicals GmbH) were slowly injected into the left atrium to delineate the 'area at risk' [17]. An overdose of potassium chloride was used to kill the animal.
2.2. Analysis of infarct size

Analysis of infarct size was performed by methods previously described in the literature [17–20] and without knowledge of the C6 status [8]. The excised heart of each rabbit was rinsed in 0.9% saline solution, weighed and cut in 2 mm slices parallel to the atrioventricular groove. The slices were photographed with a Kodak Ektachrome film to define the 'area at risk', which was the area not stained by patent blue violet. The slices were then immersed in 1% triphenyl tetrazolium chloride (TTC) in 0.2 ml Tris buffer at pH 7.8, incubated for 10 min at 37°C and rephotographed [18]. Viable myocardium was stained bright red while the region of the infarcted myocardium was demar-

Fig. 1. Impregnation of cardiac vessel walls with C5b-9 (small arrows) in a C6-competent rabbit. × 370.

Fig. 2. Intense cytoplasmic positivity of infarcted myocardial cells (small arrows) in comparison with negative immunocytochemical staining of C5b-9 in normal myocardium (large arrows). C6-competent rabbit after 2 h of coronary artery occlusion followed by 3 h of reperfusion. × 150.
cated by the absence of TTC staining. An enlarged tracing (×10) was then made from each slide and the ‘area at risk’ and the area of infarction determined by planimetry.

2.3. Area of reflow

The area of reflow into damaged myocardium was determined following a protocol recently described by Schäfer et al. [21,22]. This protocol was developed on the basis of a suggestion of Bhakdi who previously had used propidium iodide to study cell membrane permeability [23]. Propidium iodide is only able to penetrate damaged cell membranes. After intercalation with DNA it leads to a bright red fluorescence [23]. Its presence indicates that reperfusion has reached the cellular level. Other fluorescent agents have previously been employed to determine

Fig. 3. Lack of C5b-9 deposition in vessel walls (small arrows) of a C6-deficient rabbit. ×370.

Fig. 4. Negativity of C5b-9 staining in myocardial infarction in C6-deficient rabbits after 2 h of coronary artery occlusion followed by 3 h of reperfusion. ×150.
the distribution of arterial blood flow [24]. From the myocardial slices, biopsies from the area at risk as delineated by patent blue violet staining were taken and quickly frozen in n-hexane cooled to $-6^\circ$C by a mixture of dry ice in 100% ethanol and were kept at $-196^\circ$C. Cross-sections (10 $\mu$m thick) were obtained and evaluated under the microscope using fluorescent light and a filter (BP 455–500, FT 510, LP 528). The reflow into damaged myocardium (i.e., the area of fluorescent staining) was related to the whole cross-section and expressed in intervals of 10%. This analysis was performed by an independent observer who was not aware of the origin of the biopsy, the duration of occlusion and the C6 status of the animal.

The reflow into damaged myocardium expressed as a percentage of the area at risk was calculated from the results of the two biopsies.

Six biopsies were analysed by three independent observers. Their evaluations did not differ. Evaluations of the same biopsy by the same observer also did not differ when obtained several months apart.

2.4. Immunohistological studies

Adjacent cross-sections from the same material were fixed for 5 min in cooled acetone (4°C), rinsed in phosphate-buffered saline (PBS) and incubated for 2 h in a 1:20 dilution of a specific polyclonal sheep antibody against rabbit C5b-9 developed by Hugo and Bhakdi and used in a previous study [8]. After rinsing in PBS the sections were treated with the second antibody (biotinylated rabbit anti-sheep IgG) by employing a Kit (Vectastain (RJ) using a biotin avidin (ABC) bridging technique and peroxidase as a marker enzyme. Peroxidase activity was stained by 3,3'-diaminobenzidine. All processing steps were done according to the instructions of the Vectastain (R) kit.

2.5. Histological comparison of reperfused damaged myocardium and C5b-9 accumulation

C5-9 accumulation in comparison with the reperfused-damaged myocardium was assessed in adjacent cross-sections. In some cases plain histological sections were photographed under fluorescent light and subsequently subjected to immunohistological staining. This allowed direct comparison of C5b-9 accumulation and reperfused damaged myocardium in the same cross-section.

2.6. Complement titration

Serum was collected from each animal and total hemolytic activity tested by incubating 1 volume of serum with 1 volume of a 2% suspension of sheep erythrocytes coated with polyclonal antibodies (Ambozeptor 6000; Behring, Marburg, Germany) for 30 min at 37°C. C6-deficient sera were totally devoid of hemolytic activity [25].

2.7. Statistical analysis

All data are presented as mean ± SEM. Intergroup comparison of infarct size and reflow was performed by exact
permutation. Probability values of 0.05 or less were required for assumption of statistical significance.

3. Results

Forty-six rabbits were included in the experiment: 28 were C6-competent, 18 were C6-deficient. Eight of the 28 C6-competent animals died before completion of the experimental protocol: 6 from arrhythmias and 2 of other causes.

Of the remaining 20 C6-competent animals 8 were submitted to 30 min of coronary artery occlusion followed by 1 h of reperfusion in 3 animals and 3 h of reperfusion in 5 animals. Twelve C6-competent rabbits were submitted to 2 h of coronary artery occlusion followed by 1 h of reperfusion in 6 animals and 3 h of reperfusion in the 6 remaining animals. By definition, the complement titration test was always positive in C6-competent rabbits. C5b-9 depositions as revealed by immunohistological staining were always clearly visible in infarcted tissue and in vessel walls in these animals (Figs. 1 and 2).

Nine of the 18 C6-deficient rabbits were subjected to 30 min of coronary artery occlusion followed by 1 h of reperfusion in 4 animals and 3 h of reperfusion in 5 animals. The remaining 9 C6-deficient rabbits underwent 2 h of coronary artery occlusion followed by 1 h of reperfusion in 4 animals and 3 h of reperfusion in 5 animals. C6-deficient rabbits had a negative complement titration test and were devoid of immunohistochemically detectable C5b-9 accumulation (Figs. 3 and 4).

3.1. Extent and localization of the reperfused damaged myocardium within the area at risk as revealed histologically by propidium iodide staining (Fig. 5a,b and Fig. 6)

After 30 min of coronary artery occlusion followed by reperfusion the reperfused areas of the damaged myocardium as revealed by propidium iodide staining extended from the subendocardium to the mid-myocardium and often extended to the epicardium. In most cases there was a small non-fluorescent zone in the mid-myocardium. This histological appearance revealed only slight differences between C6-competent and C6-deficient animals (Fig. 5a,b).

After 2 h of coronary artery occlusion followed by reperfusion propidium iodide fluorescence was visible subendocardially and epicardially in almost all animals studied. There was a large non-fluorescent area in the mid-myocardium which was much more pronounced in C6-competent animals (Fig. 6).

3.2. Localization of C5b-9 complement accumulation in relation to the reperfused damaged myocardium (Fig. 7a,b,c)

After 30 min of coronary artery occlusion followed by reperfusion C5b-9 complement accumulated mainly at the border with the non-fluorescent area in the infarcted mid-myocardium as described above. After 2 h of coronary artery occlusion followed by reperfusion C5b-9 staining was visible throughout the infarcted myocardium.

3.3. Granulocytic infiltration of infarcted tissue in C6-competent and C6-deficient animals

Infarcted areas presented with a beginning slight and inhomogeneous granulocytic infiltration without differences between the C6-deficient and C6-competent groups (data not shown).

3.4. C6 status and infarct size (Fig. 8)

Only animals that were subjected to 3 h of reperfusion were considered in the final analysis of infarct size to
allow sufficient time for complete infarct size delineation by TTC staining [27].

After 30 min of coronary artery occlusion followed by 3 h of reperfusion infarct size (TTC negative area) expressed as a percentage of the ‘area at risk’ (patent blue-violet negative area) was significantly smaller in C6-deficient rabbits (5.0 ± 2%) than in C6-competent rabbits (28.4 ± 8.5, P = 0.0371).

After 2 h of coronary artery occlusion followed by 3 h of reperfusion infarct size did not differ between C6-deficient and C6-competent animals.

As expected, infarct size increased with the time of

Fig. 7. (a) Middle section of Fig. 5 at higher magnification. Collage, × 25. (b) Same cross-section as Fig. 7a stained immunohistologically for C5b-9. Note the positivity of C5b-9 staining at the border with the non-fluorescent area. Collage, × 25. (c) Area from same slide (large arrow) at a higher magnification (Magnification × 370).
coronary artery occlusion, suggesting that after 30 min of coronary artery occlusion the infarct was not complete.

3.5. C6 status and area of reflow (Fig. 9)

For the evaluation of the area of reflow, differences in the duration of reperfusion (1–3 h) were ignored because they have no influence on the area of reflow and C5b-9 complement accumulation [8,21,22].

Three animals had to be excluded because the amount of material obtained was not sufficient for evaluation.

After 30 min of coronary artery occlusion followed by reperfusion the area of reflow (positive propidium iodide fluorescence) expressed as a percentage of the 'area at risk' was nearly the same in both animal groups (38 ± 9% in C6-deficient rabbits vs. 39 ± 7% in C6-competent rabbits; P = 0.5722, n.s.).

After 2 h of coronary artery occlusion followed by either 1 or 3 h of reperfusion the area of reflow was significantly smaller in C6-competent rabbits (25 ± 4) than in C6-deficient animals (40 ± 4; P = 0.0185).

Thus, in contrast to infarct size, a statistically significant difference in reflow between C6-deficient and C6-competent animals could only be detected after 2 h of coronary artery occlusion followed by reperfusion.

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Fig. 8. Infarct size in C6-competent rabbits (solid bars) and C6-deficient rabbits (open bars) after 30 min and after 2 h of coronary artery occlusion followed by 3 h of reperfusion. Note the significantly smaller infarct size in C6-deficient animals after 30 min of coronary artery occlusion.

Fig. 9. Area of reflow in C6-competent rabbits (solid bars) and C6-deficient rabbits (open bars) after 30 min and after 2 h of coronary artery occlusion followed by 3 h of reperfusion. Note the significantly larger area of reflow in C6-deficient animals after 2 h of coronary artery occlusion.
In contrast to C6-competent animals whose reflow declined with the time of coronary artery occlusion, in C6-deficient animals reflow into damaged myocardium was nearly the same after 30 min and 2 h of coronary artery occlusion followed by reperfusion in C6-deficient animals.

3.6. Correlation between reflow and infarct size (Fig. 10a,b)

There was no correlation between reflow and infarct size either in C6-competent or in C6-deficient animals after shorter and longer coronary artery occlusions.

Fig. 10. Absence of any correlation between reflow and infarct size after coronary artery occlusion followed by reperfusion. (open symbols: C6-deficient animals; closed symbols: C6-competent animals). (a) 30 min of coronary artery occlusion. (b) 2 h of coronary artery occlusion.
3.7. Arrhythmia profile (Fig. 11)

Seven of the 18 C6-deficient animals had arrhythmias. All of them occurred during coronary artery occlusion. Five animals suffered from minor arrhythmias (Lawn I-II). The remaining 2 animals experienced major arrhythmias (Lawn III-IV); none of these was fatal.

Of the 28 C6-competent rabbits 16 experienced arrhythmias: 13 occurred during coronary artery occlusion, 3 after reperfusion was initiated; 5 animals had minor arrhythmias and 11 animals had major arrhythmias. In 6 cases these arrhythmias resulted in the death of the animal.

3.8. Timing of arrhythmias (Table 1)

Light arrhythmias occurred shortly after ligation and sometimes continued throughout coronary artery occlusion. There was no difference in the incidence between C6-competent and C6-deficient rabbits. Severe arrhythmias in C6-deficient rabbits also occurred shortly after ligation of the coronary artery. In C6-competent rabbits, however, severe arrhythmias more often occurred later after coronary artery occlusion and then tended to be fatal.

Thus C6-deficient animals suffered from fewer and less severe arrhythmias than C6-competent rabbits. Most of the

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<td>C6-status</td>
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<td>No. of animals experiencing only light arrhythmias (Lawn I-II)</td>
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<td>No. of animals experiencing severe arrhythmias (Lawn III-IV)</td>
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Note that more C6-competent rabbits experienced severe and lethal arrhythmias more than 30 min after coronary artery occlusion. C = C6-competent; D = C6-deficient.
Arrhythmias occurred after coronary artery occlusion. Severe arrhythmias with fatal outcome only occurred in C6-competent rabbits. The onset of these fatal arrhythmias was later after coronary artery occlusion. Only 3 C6-competent animals presented with reperfusion arrhythmias.

4. Discussion

The major aim of the present investigation was to determine whether the terminal complement sequence (i.e., formation of C5b-9) might additionally contribute to reperfusion tissue damage. This question appeared significant in view of the fact that C5b-9 complement complexes accumulate during myocardial infarction in humans and in rabbits, and that C5b-9 deposition appears to occur during the reperfusion phase [8]. We elected to perform experiments using C6-deficient rabbits because these animals totally lack C6; thus, complement activation leads to normal production of C5a but not to production of C5b-9. In most experiments, paired homozygote and heterozygote siblings from the same litter were employed. The general morbidity and proneness to infection were not higher in C6-deficient rabbits. Additional macroscopic and histological examinations of other organs did not reveal any differences between C6-competent and C6-deficient rabbits. Experiments were performed in a blinded fashion and myocardial infarct sizes and reperfusion areas were documented without knowledge of the C6 status.

Our results suggest that cytotoxic C5b-9 complex contributes to reperfusion injury after short coronary artery occlusions. We were able to exclude that this was merely an effect of patchy infarction in the C6-deficient group using propidium iodide as a histological marker of cell damage and reperfusion. In both groups there were large areas of propidium iodide fluorescence often interrupted by a central zone of absent fluorescence which corresponds to a central zone of no-reflow previously described [24]. C5b-9 complement mainly accumulated at the border of this zone.

After 2 h of coronary artery occlusion the infarct sizes, however, did not differ between C6-competent and C6-deficient animals. Other groups have shown that the infarct is complete in the rabbit heart at this time [26–28]. Our results therefore confirm that C5b-9 complement rather contributes to reperfusion injury than to ischaemic tissue damage in the non-reperfused heart. The small infarct sizes observed in our study are probably due to the shrinkage of tissue in the TTC solution which others have also observed in their experiments (Schaper, personal communication). Since the area at risk is delineated before TTC staining, this leads to an underestimation of infarct size in percentage terms. Thus, however, is a systematic error applicable to all groups studied and still permits comparison of infarct sizes. Propidium iodide staining at the end of reperfusion revealed that C5b-9 contributes to the ‘no-reflow’ phenomenon after 2 h of coronary artery occlusion when no difference in infarct size was observed. Our results also show that there is no correlation between infarct sizes and reflow. This is in accordance with the observations of Darsee and Kloner and Fishbein et al. who have shown that any area of microvascular injury resulting from reperfusion lies within the area of the infarct and therefore is not a causative factor in increasing infarct size [29,30]. Propidium iodide staining did not render an explanation for the smaller infarct sizes of C6-deficient animals after short coronary artery occlusions. This fluorophore, however, only stains the reperfused damaged myocardium. After 2 h of coronary artery occlusion when infarction is complete, propidium iodide labeling therefore renders a very good estimate of the area of reflow. After 30 min of coronary artery occlusion, however, we cannot exclude the possibility that the propidium-iodide-negative areas contain non-perfused as well as viable areas even though we had already seen epicardial propidium iodide staining. This may have blunted a possible effect of C5b-9 on the ‘no-reflow’ phenomenon after short coronary artery occlusions.

In spite of the rapid accumulation of C5b-9 during reperfusion this does not lead to a greater incidence of reperfusion arrhythmias which we only saw in 3 cases. The ability to form C5b-9 surprisingly seems to influence arrhythmogenesis after coronary artery occlusion. Severe arrhythmias were mainly observed after 30 min of coronary artery occlusion in C6-competent rabbits. These ‘late-occurring’ arrhythmias tended to be fatal. At this time complement accumulates in small amounts at the border zone of the infarcted tissue, as our group has previously described [8]. Border zones have been implicated to play an important role in the generation of a re-entry mechanism which is thought to be responsible for arrhythmogenesis after myocardial infarction [31,32]. The ability of nucleated cells to withstand an attack of lytic complement might also lead to transient alterations of membrane potentials of myocytes at the border zone [14,33]. Thus, the higher incidence of arrhythmias observed in C6-competent rabbits might be due to facilitated automaticity and re-entry.

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