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ORIGINAL ARTICLE

C. Ortmann · H. Pfeiffer · B. Brinkmann

Demonstration of myocardial necrosis in the presence of advanced putrefaction

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Abstract Samples of heart tissue were investigated in two series for the detectability of myocardial necrosis after artificial and natural putrefaction, respectively. In the first series heart tissue with and without infarction was artificially subjected to humid and dry autolysis and putrefaction. In the second investigation heart tissue was obtained from exhumed bodies after periods of burial ranging between 10 and 929 days. Besides histology a variety of immunohistochemical markers were applied and C5b-9 gave positive results even after long periods of artificial and natural putrefaction. From the methods tested, this was by far the most sensitive method with a high robustness against putrefaction. NP57, which indicates neutrophilic leucocytes could be demonstrated considerably longer after humid putrefaction than after dry putrefaction. The time limits of detection were considerably longer than for H&E. These two methods are the methods of choice for the detection of myocardial infarction and leucocyte infiltration in advanced stages of putrefaction.

Key words C5b-9 · NP57 · Putrefaction · Autolysis · Myocardial infarction

Introduction

Autopsies carried out after exhumation of a human body are the last resource to investigate the cause of death. Questions to be addressed concern the medical treatment, insurance aspects as well as a delayed suspicion of a crime. In many cases, mismanagement during the investigations of the circumstances surrounding the death seems to be the major reason for exhumations (Brinkmann et al. 1986; Banaschak et al. 1998). Due to putrefaction and autolysis the diagnostic specificity can be substantially restricted and thus the number of differential diagnoses is increased. In particular, the appearance of acute myocardial infarction (AMI) can be masked or even imitated by autolysis and putrefaction (Knight 1973; Fechner and Sivaloganathan 1987; Leadbetter et al. 1989).

Although selected reports have dealt with the sporadic demonstration of related changes after long time periods of burial, such as thrombosis of coronary arteries (118 or 166 days Althoff 1974; Seibel et al. 1997), calcified coronary arteries and myocardial scars (1581 days Seibel et al. 1997), leucocyte infiltration (30 days Vock 1986) such observations are either non-specific for the cause of death or are the exception.

We have therefore introduced putrefaction resistant markers of early infarction and related leucocyte infiltration by improved immunohistochemistry (IH) and evaluated the diagnostic significance.

Material and methods

Experiment 1

Heart tissue was obtained from three cases of extended acute myocardial infarction (AMI) and from three acute deaths due to hanging. Autopsies were performed 1–2 days after death. The hearts were cut into small pieces (0.4 × 1 x 1 cm) placed in tissue cassettes and incubated either in the blood of the donor or in an open vessel at room temperature (16–20 °C). Specimens from AMI heart tissue were taken from the marginal zone of AMI thus containing infarcted and non-infarcted myocardium and incubated for up to 8 weeks. After each week, one specimen from each series was selected for further investigation. Before fixation, air dried specimens were subjected to rehydration with a 24 h incubation in tap water. The specimens incubated in blood were first washed under flowing tap water. After fixation (24 h in 4% buffered formalin) the samples were processed as normal and embedded in paraffin.

Experiment 2

Samples were obtained from 26 exhumations and formalin-fixed and paraffin-embedded myocard tissue was analysed for the markers listed in Table 1). The length of burial was up to 929 days before exhumation (Tables 2 and 3). Six cases with acute causes of death and only low grade coronary arteriosclerosis were used as

C. Ortmann (✉) · H. Pfeiffer · B. Brinkmann
Institut für Rechtsmedizin, Westfälische Wilhelms-Universität,
Von-Esmarch-Strasse 62, 48129 Münster, Germany
Fax: +49-251-8355158

Table 1 Incubation times and antibodies used in this study

Antibody against	Autoclave pre-treatment	Proteinase K pre-treatment	Incubation time of primary antibody, temperature	Concentration of primary antibody
Fibrinogen (DAKO)	–	–	60 min, 20 °C	1 : 500
Fibronectin (DAKO)	–	30 min	30 min, 20 °C	1 : 100
Desmin (Sigma)	+	10 min	60 min, 20 °C	1 : 40
Myoglobin (DAKO)	+	10 min	60 min, 20 °C	1 : 100
FABP heart type	+	10 min	60 min, 20 °C	1 : 100
Troponin C human cardiac (Novo Castra)	+	–	60 min, 20 °C	1 : 100
C5b-9 (DAKO)	+	10 min	60 min, 20 °C	1 : 50
NP57 (DAKO)	–	–	30 min, 20 °C	1 : 50

Table 2 Details of the control cases after exhumation and the results of C5b-9 and NP57 with diffusely scattered positive cells

Time between death and autopsy (days)	Season of death	Sex	Age (years)	Cause of death	C5b-9 positive myocytes	NP57
16	Summer	F	52	Strangulation	–	+
209	Summer	M	26	Polytrauma	–	+
211	Summer	M	34	Polytrauma	–	+
252	Summer	M	32	Polytrauma	–	–
301	Spring	M	30	Cerebral bleeding	–	+
929	Spring	M	50	Polytrauma	–	–

Table 3 Exhumation cases with presumed acute cardiac injury (C5b-9: + positive single myocytes, ++ greater areas of positive reaction; NP57: + diffusely scattered, ++ strong infiltration)

Time between death and autopsy (days)	Season of death	Sex	Age (years)	Cause of death	C5b-9 positive myocytes	NP57
10	Summer	M	52	Unknown	–	+
16	Spring	M	61	Coronary insufficiency	+	+
16	Winter	M	49	Unknown	–	+
20	Summer	F	51	Unknown	–	+
23	Winter	M	56	Coronary insufficiency	+	–
31	Summer	M	46	Coronary insufficiency	–	+
52	Spring	F	72	Gastrointestinal bleeding	–	+
63	Spring	M	45	Coronary insufficiency	++	+
78	Spring	M	54	Unknown	–	+
122	Spring	F	77	Unknown	–	+
128	Summer	M	71	Multiple organ failure after heart valve operation	++	++
148	Autumn	F	47	Unknown	–	+
175	Winter	M	68	Unknown	+	–
203	Spring	M	65	Coronary insufficiency	–	+
236	Winter	M	61	Coronary thrombosis	+	+
242	Winter	M	66	Unknown	+	+
269	Spring	F	73	Unknown	–	+
284	Spring	M	74	Pneumonia	++	+
487	Winter	F	62	Pneumonia	+	+
807	Spring	M	53	Unknown	–	–

exhumation controls (Table 2). In the remaining cases, there was a variety of findings indicating the possibility of acute myocardial damage i.e. clinical records, advanced coronary arteriosclerosis or myocardial scars or thrombosis of a coronary artery (one case, Table 3).

Methodology

Pre-treatment procedures for immunhistochemistry (IH) were carried out as described previously (Ortmann and Brinkmann 1997). Negative controls were included without primary antibody and were taken from serial sections. Possible endogenous biotin (DAKO biotin blocking system), alkaline phosphatase (Levamisole) or peroxidase (DAKO peroxidase-blocking solution) were blocked before the corresponding IH was carried out.

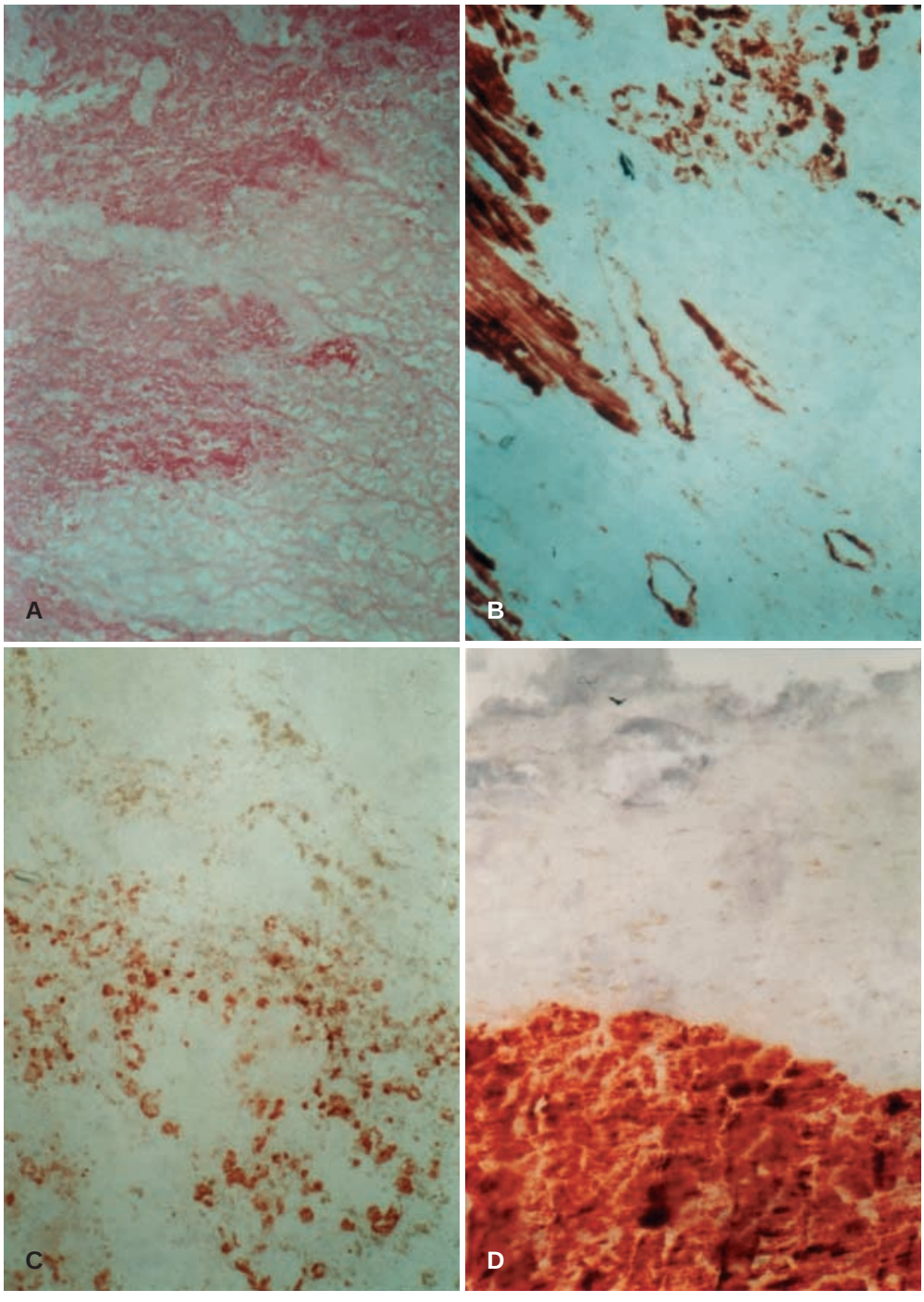

Fig. 1 AMI with experimentally induced putrefaction and autolysis after 3 weeks incubation in blood **A** and air **B** and after 7 weeks incubation in blood **C** and air **D**

For C5b-9 a biotinylated secondary antibody was employed and for visualisation streptavidin-conjugated alkaline phosphatase and neufuchsin (SAPN). Alternatively, C5b-9 was detected with streptavidin-conjugated peroxidase and 3-amino-9-ethylcarbazole (SPAEC).

NP57 is a monoclonal antibody against human neutrophil elastase (Pulford et al. 1988) and was used for the detection of granulocytes with a biotinylated secondary antibody, streptavidin-conjugated peroxidase and AEC (SPAEC). In experiment 2, C5b-9 was only investigated with both methods asmentioned when the SAPN method gave positive results.

Serial sections of all specimens were also stained for two plasma antigens (fibronectin, fibrinogen) and four structural antigens (desmin, troponin C, FABP, myoglobin) as described previously (Ortmann et al. 2000, Table 1). Controls were processed accordingly.

Serial sections were additionally routinely stained with haematoxylin eosin (H & E).

Results

Experiment 1

Before exposure to autolysis and putrefaction, all heart sections gave clear results according to the specificity of

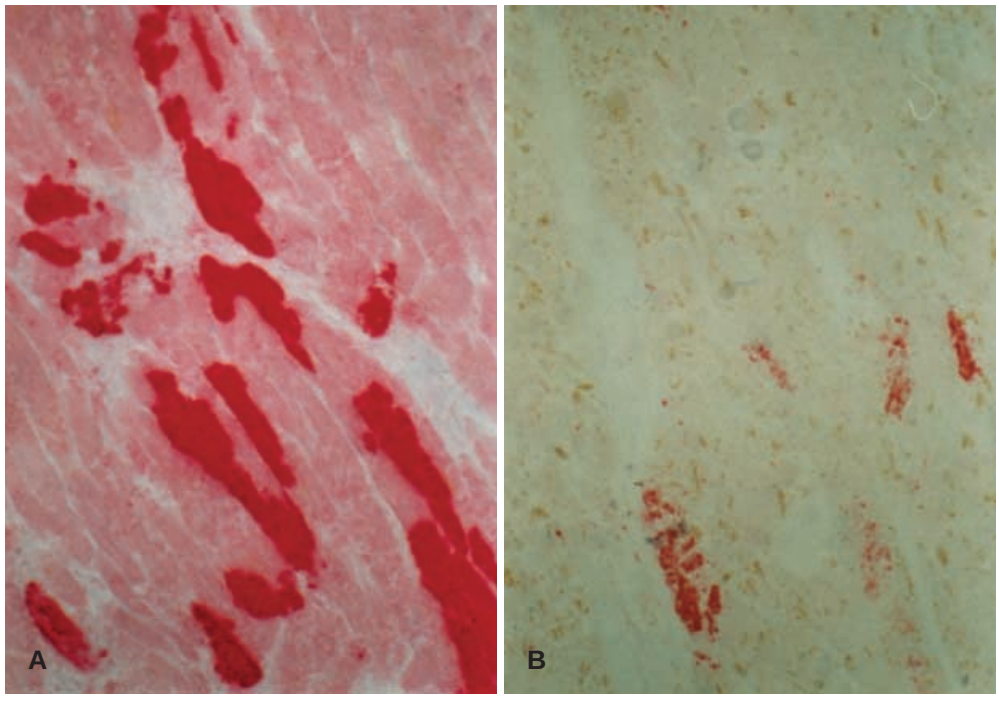

Fig. 2 C5b-9 positive single cell necrosis after 63 days **A** and 487days **B** between death and autopsy. Note diffuse background staining in **A**

the methods applied. The changes caused by the same incubation method were relatively uniform and varied little between the specimens.

Incubation time of 1 week

– Histology (H&E): Shrunken myocardium with pyknotic nuclei and good detectability of leucocytes after air incubation; widely spaced muscle fibres with mostly invisible nuclei, poor and reduced detectability of leucocytes, some gas bubbles after blood incubation; bacterial colonisation and fungal mycelia in both series; no detectability of AMI.

– Plasma and structure antigens: Both types of antigens gave a weak and diffuse staining intensity in areas with and without necrosis and the structure antigens showed strong depletion in non-infarcted areas. No significant difference between both incubation methods.

– NP57: After air incubation leucocytes gave a negative reaction although still positive with H&E. After blood incubation no reduction of intensity and cell number.

– C5b-9: Good and clear staining of infarcted areas in both series. Staining intensity after air incubation weaker.

Incubation over subsequent weeks

– H&E and plasma and structural antigens: The intensity of the reaction faded out.

– NP57: After blood incubation up to 3–4 weeks, there was no significant loss of intensity and cell numbers but after 4 weeks cell numbers decreased. After 7–8 weeks only single cells were detected. No non-specific staining.

– C5b-9: After blood incubation unequivocal reactions up to 2–3 weeks (Fig. 1a) but increasing degrees of disintegration of myocytes and later, patchy detection of weakly to moderately stained in almost totally effaced structures (Fig. 1 c). After air incubation the staining intensity became gradually weaker but the reaction remained the same over the 8-week period (Fig. 1 b, d). Blood incubation was associated with a non-specific uniform background reaction. “Positive” structures, i.e. positive arterial walls and/or necrotic myocardium were still easily discernible against the background.

SAPN showed higher background staining but also a higher sensitivity compared to SPAEC.

Experiment 2

Control cases

C5b-9 positive reactions of the arterial walls were detected but no positive reaction of the myocardium and no artefacts. Isolated NP57 positive cells were diffusely scattered in four cases and two cases were NP57 negative. There was no unspecific background staining and no unspecific staining of other cells.

Indication of ischemic heart disease (IHD 20 cases)

In nine cases (45%) C5b-9 positive single cell necrosis or necrosis of small groups of muscle fibres could be de-

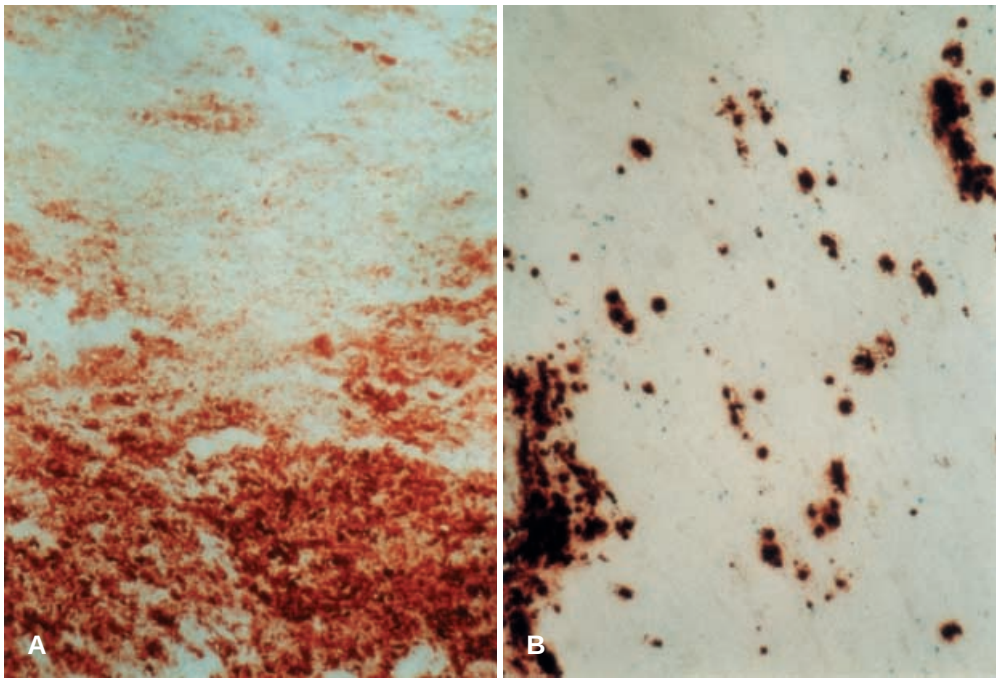

Fig. 3 A, B A patient who died some days after a heart valve operation and acute cardiac insufficiency in a clinically diagnosed multiple organ failure. The time between death and autopsy was 128 days. Remnants of C5-9 positive myocytes **A** and in the same region infiltration of NP57 positive cells **B** can be seen

tected and in some cases (3 cases) the areas of reaction were larger. In two cases heavy background staining was found for SAPN but not for SPAEC, but SAPN showed a higher staining intensity and therefore a higher sensitivity. All other IH markers and H&E gave negative staining results for the detection of infarction. The highest level of disintegration of myocytes was observed in a case with a burial time of 128 days and in this case residual areas with a strongly positive C5b-9 reaction were observed (Fig. 3 A) which together with an infiltration of NP57 positive cells (Fig. 3 B) led to the diagnosis of relevant necrosis. Three further cases were completely negative for NP57 (Table 3).

Discussion

C5b-9 has been demonstrated to have a high specificity for the detection of myocardial infarction (Thomsen and Held 1995). In previous experiments we observed a rapid decrease of IH markers in the early stages of autolysis including C5b-9 (Brinkmann et al. 1993). Another group employing the APAAP method of detection observed positive reactions up to the 11th day of the putrefaction process (Thomsen and Held 1994). In our present series the demonstration of this marker over time was very much extended. We suggest that this is due to two reasons, i. e. the application of much more sensitive methods (SAPN, SPAEC) in conjunction with better antigen retrieval techniques (Edston and Kawa 1995; Pileri et al. 1997). Both these factors together indicate that C5b-9 is at present the most suitable antigen for the detection of early infarction, by combining high specificity and sensitivity and robustness against postmortem changes. There exist of course a variety of methods which give a positive reaction in the earlier phases after infarction (Amberg 1995; Kleine et al. 1993; Ortmann et al. 1999) but are less specific and less robust (Fieguth et al. 1997). In the present study much earlier deterioration of these markers was found in the early putrefaction period.

NP57 reacts with neutrophil elastase which has a function similar to C5b-9 in early cell injury and defence (Pulford et al. 1998). It lies predominantly in the azurophilic granules of neutrophils and in smaller quantities in some monocytes (Dewald et al. 1975). The present study has shown that after blood incubation, NP57 can be detectable for several weeks and detection decreases much earlier after air incubation. It also needs to be stressed that autoclave pre-treatment inhibited the detection of NP57. Especially when compared with conventional histology (H & E), NP57 was found to be a much more robust marker during the putrefaction process but a clear infiltration was found in only one case with presumed cardiac injury (Table 3).

It is well known that the putrefaction process depends on a variety of factors and therefore the extent of putrefaction correlates poorly with the length of the burial period. Also, the time periods as defined experimentally cannot be extrapolated to exhumation cases. Therefore the results do not yet allow an exact prediction of the sensitivity of our methods in unknown cases but if myocardial necrosis is present and remnants of the necrotic area can be detected then this is at least the method of choice.

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