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The application of selected histochemical and immunohistochemical markers and procedures to the diagnosis of early myocardial damage

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Summary. Histochemical (= HIS) methods (haematoxylin-eosin, luxol fast blue, chromotrope aniline blue) and various immunohistochemical (= IH) markers (myoglobin, desmin, fibrinogen, complement C5b-9) were applied in parallel to test the efficiency, specificity and sensitivity for the recognition of early ischemic myocardial damage. The whole series was subgrouped into cardiac deaths (N= 35) and controls (N = 13). Cardiac deaths were sub-divided into 3 groups: 1. infarction visible in gross examination (N = 15), 2. coronary thrombosis without infarction (N = 11), 3. stenosing coronary athero-sclerosis without infarction (N = 9). The control group (group 4) consisted of unnatural deaths with presumed short agonal periods (N = 13). Group 1 cases usually exhibited extended coagulation necrosis of the diffuse type and the contraction type in combination (1 exception). Group 2 showed mainly a patchy type of coagulation necrosis and contained 1 case where all methods failed to react and 3 more cases where only the HIS methods failed to react. Group 3 and 4 were associated with a disseminated type of single and/or grouped fibre necrosis. – In addition, the average reaction strengths showed a decrease from group 1 to group 4 which was more pronounced in the HIS reactions compared with the IH reactions. One case in group 1 showing negative IH reactions cannot be explained. Positive IH reactions observed in a few cases in group 2 contrasting with negative HIS reactions would indicate a greater sensitivity of this methodology and this interpretation also applies to groups 3 and 4. From pathophysiological considerations, the positive cases in groups 3 and 4 can be well explained. – The results show that selected application of a single criterion to the diagnosis of early myocardial infarction and/or ischemic fibre damage cannot resolve the diagnostic problem. However, a selected set of HIS/IH methods and the synoptic interpretation of all findings will improve the detection of early myocardial infarction/ischemic damage.

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Key words: Myocardium – Ischemic damage – Histochemistry – Immunohistochemistry

Zusammenfassung. Histochemische (= HIS) Methoden (Hämatoxylin-Eosin, Luxol Fast Blue, Chromotrop Anilin-Blau) und verschiedene immunhistochemische (= IH) Marker (Myoglobin, Desmin, Fibrinogen, Complement C5b-9) kamen nebeneinander zur Anwendung, um die Effizienz, Spezifität und Empfindlichkeit zur Erkennung früher ischämischer Herzmuskelschäden zu überprüfen. Das Untersuchungskollektiv war unterteilt in Herztodesfälle (N = 35) und Kontrollen (N = 13). Die Herztodesfälle waren in drei Gruppen unterteilt: 1. Makroskopisch erkennbarer Infarkt (N = 15), 2. Koronarthrombose ohne Infarkt (N = 11), 3. stenosierende Koronararteriensklerose ohne Infarkt (N = 9). Die Kontrollgruppe (4.) bestand aus nicht-natürlichen Todesfällen mit vermuteten kurzen Agonie-Zeiten (N = 13). Die Fälle der Gruppe 1 zeigten normalerweise eine ausgedehnte Koagulationsnekrose mit einer Kombination des diffusen Typs und des Kontraktionstyps (eine Ausnahme). Die Fälle der Gruppe 2 zeigten einen herdförmigen Typ der Koagulationsnekrose. Sie enthält ferner einen Fall, in welchem alle Methoden versagten und drei Fälle, in welchen die HIS Methoden versagten. Positive Fälle der Gruppen 3 und 4 zeigten einen disseminierten Typ einzelner oder gruppierter Fasernekrosen. – Zusätzlich zeigten die durchschnittlichen Reaktionsstärken eine Abnahme von Gruppe 1 nach Gruppe 4. Diese Abnahme war in den HIS Reaktionen ausgeprägter als in den IH Reaktionen. Ein Fall der Gruppe 1 mit negativer IH findet keine Erklärung. In einigen Fällen der Gruppe 2 wurden positive IH Reaktionen bei negativen HIS Reaktionen beobachtet, was auf eine größere Empfindlichkeit der IH Methodologie hinweist; diese Interpretation gilt ebenfalls für die Gruppen 3 und 4. Aus pathophysiologischen Überlegungen lassen sich die positiven Fälle der Gruppen 3 und 4 gut deuten. – Die Ergebnisse zeigen, daß die isolierte Anwendung eines einzelnen Kriteriums zur Diagnostik des frühen Myokardinfarkts und/

oder des ischämischen Faserschadens dieses diagnostische Problem nicht lösen können. Jedoch wird ein ausgewählter Satz von HIS/IH Methoden sowie die synoptische Interpretation aller Befunde die Erkennbarkeit des frühen Myokardinfarkts/ischämischen Schadens verbessern.

Schlüsselwörter: Myokard – ischämischer Schaden – Histochemie – Immunhistochemie

Introduction

The demonstration of early ischemic lesions in the myocardium has been the subject of many investigations. Using conventional histology, the time of manifestation is usually in the range 5–8 hours (Riede and Drexler 1989). – Electron microscopy seems to offer many advantages, but early autolysis can simulate and overlay subcellular changes caused by other mechanisms and thus mask them (Hackel and Reimer 1990). Many researchers have applied enzyme histochemistry, i.e. mainly the visualization of dehydrogenases with different dyes (Nachlas and Shnike 1963; Andersen and Hansen 1973; Derias and Adams 1978; Becker and Andersen 1985) but here also many artefacts can be caused by autolysis (Caesar 1984). – In addition, several histochemical reactions have been introduced, e.g. so-called fuchsinophilia and fuchsinorrhagia (Lie et al. 1971; Knight 1979), UV-fluorescence (Carle 1981; Badir and Knight 1987). Other enzymes have also been studied, e.g. phosphorylase (Janssen 1977; Caesar 1984). Also, the measurement of the ratios of potassium/sodium in tissues has been reported to be a specific test (Saukko 1983). However, most of the aforementioned methods are either non-specific (failure to differentiate between autolysis and ischemic lesions) or the changes induced by ischemia occur too late (Hackel and Reimer 1990). More recently, new histochemical methods have been proposed, e.g. a modification of luxol fast blue (LFB) by Arnold et al. (1982, 1985, 1986) and chromotrope aniline blue trichrome staining (CAB) by Zollinger (1989). Even more recently, a series of immunohistochemical markers of early myocardial lesion has been reported by various authors (Schäfer et al. 1986; Leadbetter et al. 1989, 1990; Shekonin et al. 1990; Thomsen et al. 1990; Chumachenko and Vikhert 1991).

This study attempts to simultaneously apply a set of these methods and compare their efficiency, specificity and sensitivity for the detection of early myocardial lesions.

Materials and methods

A total of 48 hearts were systematically studied of which 35 were cardiac deaths and 13 were controls: 4 subgroups were formed (Table 1):

- 1/inf. = *inf*arction macroscopically present ($N = 15$)
- 2/thr. = occlusive coronary *thr*ombosis without infarction ($N = 11$)
- 3/ath. = coronary *ath*erosclerosis without infarction/thrombosis ($N = 9$). To fulfill this definition, extensive other investigations were necessary, i.e. histology and toxicology and the minimal degree of stenosis required in the proximal arteries was 75%. A further differentiation of the degree of stenosis was not carried out. In group 2 and 3 macroscopical investigation revealed the presence of very small disseminated areas of scar tissue but no larger infarct scars or aneurysms could be detected.
- 4/con. = the *con*trol group ($N = 13$) consisting of deaths due to other mechanisms with known agonal periods (Table 2)

Cases in which resuscitation using catecholamines and/or defibrillation had been attempted were not included and also those hearts were rejected which showed signs of incipient putrefaction either in gross examination or in H&E sections. The postmortem intervals varied between 6 and 144 hours and did not significantly differ between subgroups 1–4. The histology specimens were taken from the following areas:

- 1/inf.: periphery of the infarction zone and adjacent myocardium without macroscopical changes
- 2/thr.: at least 2 samples from areas presumably involved and 1 control specimen
- 3/ath.: same as group 2/thr.
- 4/con.: specimens from anterior and posterior wall of the left ventricle and the septum

In 3 cases additional specimens from the same areas as described were placed in an incubator at 20°C and small sections were removed at intervals of approximately 2–3 days up to 8 days (Fechner and Sivaloganathan 1987). – Specimens were fixed in 4% buffered formalin, embedded in paraffin and 5 µm sections were made. Using indirect immunohistochemistry (IH) the following antigens were investigated:

Primary antibody. Polyclonal rabbit; anti human antibodies against desmin, myoglobin, fibrinogen; dilution 1/50; Dako, D-22047 Hamburg.

Table 1. Distribution of age, sex, survival period, postmortem time and heart weight in the groups investigated

Group	*N*	Sex		Age (years)	Survival[a] period (h)	p.m. time[b] (h)	heart weight (g)
		m	f				
1	15	13	2	26 –85	0–80	214– 96	330–700
2	11	10	1	27 –83	0–15	6– 96	320–710
3	9	6	3	42 –91	0–24	5–144	380–460
4	13	9	4	10w–51	0– 1	7– 48	220–490

[a] The exact time of commencement of ischaemia could not be determinded in every case
[b] Time between death and autopsy

Table 2. Causes of death in the control group (group 4)

Cause of death	Number of cases	Case-no.
Gunshot	3	41, 42, 46
Drowning	2	40, 44
SIDS	2	37, 47
Hanging	1	43
Heroin	1	38[a]
Carbon monoxide	1	39[b]
Head injury	1	36
Multiple trauma	1	45
Bleeding	1	48

[a] 186 ng morphine/g blood
[b] carbon monoxide concentration: 75%

For C5b-9 complement-complex (C5b-9): anti-human C5b-9; polyclonal rabbit; dilution 1/10; Calbiochem, D-65812 Frankfurt/M.

Secondary antibody. For myoglobin and fibrinogen: swine immunoglobulin to rabbit immunoglobulin; dilution 1/50; Dako, D-22047 Hamburg.

For desmin: biotinylated rabbit immunoglobulin to sheep/mouse immunoglobulin; dilution 1/500, Boehringer, D-6800 Mannheim.

For C5b-9 comp.: biotinylated rabbit immunoglobulin to mouse immunoglobulin; dilution 1/300, Dako, D-22047 Hamburg.

Third step. For myoglobin and fibrinogen: PAP-complex, Dako, D-22047 Hamburg.

For desmin: Streptavidin-complex, Boehringer, D-6800 Mannheim.

For C5b-9 comp.: Avidin-Biotin-complex, Dako, D-22047 Hamburg.

Substrates. Myoglobin, fibrinogen and C5b-9 comp.: 3-Amino-9-ethylcarbazole (AEC), Sigma, D-8024 Deisenhofen.

Desmin: 3,3-Diaminobenzidine-tetrahydrochloride (DAB), Amersham, United Kingdom.

We used the following structures as positive controls: normal human myocardial fibres for desmin and myoglobin; for fibrinogen its presence in vital haemorrhages (Berg 1975); for C5b-9 comp. its presence in vital muscle lesions (Fechner et al. 1993). Structures that are known to lack the relevant antigens served as negative controls. Furthermore, in each experiment one reagent was omitted from the necessary components, e.g. the primary antibodies.

Sections from all specimens were also stained using haematoxilin and eosin (H&E), a modification of luxol fast blue (LFB, Arnold et al. 1985) and chromotrope aniline blue (CAB, Zollinger 1983). The H&E sections were also examined using UV fluorescence in addition to light microscopy. In the following, the histological/histochemical part is abbreviated HIS and the immunohistochemical one IH.

All sections were investigated and evaluated by 2 independent examiners. The results were laid out in the form of a table using the following major parameters:

- H&E – coagulative necrosis (HEFd – *d*iffuse type of eosinophilia; UV-*F*luorescence
  – *c*ontraction band necrosis (HEFc)
- LFB – *d*iffuse type of myofibrillar degeneration (LEBd)
  – *cr*oss band type of myofibrillar degeneration (LFBcr)
- CAB – contraction band necrosis (CABc)
- desmin/myoglobin – loss or depletion of antigens, displacement of these proteins within fibres
- fibrinogen/C5b-9 – positive reaction relative to relevant structures

The strength of the reactions and/or their extent were scored semiquantitatively using a 4-degree scale: 0 = negative, 1 = slight, 2 = moderate, 3 = extensive.

Results

The histological/histochemical reactions showed 2 different types: (1) "positive" reactions with an enhancement of the intensity or with the occurrence of other colourations and/or in combination with other patterns/changes, e.g. contraction bands. This type was associated with HE, LFB, CAB, F, C5b-9, whilst (2) the other type associated with D and M showed a fading. The fibrinogen reaction was usually much stronger and more intense than that for C5b-9 comp. and the topographical distribution was similar to that of the HIS alterations (Figs. 1–2).

These associations were regularly observed in the infarction group and became more irregular from group 2 through group 3 to group 4.

Group 1/inf (Figs. 3, 4): the most pronounced changes were observed in the marginal zone of infarction. The areas involved were either patchy or extensive. All 5 histochemical parameters were present and the scores were usually evenly distributed. The immunohistochemical (IH) parameters D (= desmin), M (= myoglobin) and F (= fibrinogen) reacted very much in parallel, while C5b-9 comp. was more often negative. There was only one case (No. 1), where all HIS parameters were only slightly positive and the IH reaction failed to react.

Group 2/thr (Figs. 3, 4): the extent of the areas involved was usually patchy and the average reaction scores were much weaker than in the aforementioned group (ca. 50%). In one case, where all parameters were negative and in 3 more cases where only 2–3 out of 9 reacted, the negative reactions were observed for all HIS parameters.

Group 3/ath (Figs. 3, 4): the patchy type of alterations was only observed in one case (number 32) while all other cases showed a disseminated type of single fibre or grouped fibres reaction. The quantitative scores were again reduced while the immunohistochemstry remained more or less unchanged (when compared to the aforementioned group). All reactions failed in one case while 3 other cases showed only 2–3 positive reactions.

Group 4/con (Figs. 3, 4): although on average there was a further steep decrease of the reaction strengths, 11 out of 13 cases showed the disseminated type of single cell alterations. The HIS parameters and the C5b-9 comp. were mainly negative, showing only 5 positive reactions in 4 cases while the IH (D, M, F) revealed a higher frequency of positive reactions.

Autolysis. The influence of autolysis was tested in 2 cases (Fig.5) and showed a rapid decrease of positive reactions over the time period tested. A strong reaction pattern in the early postmortem period was converted into a moderate reaction after 2–3 days and the disappearance of all markers occurred after approximately 1 week.

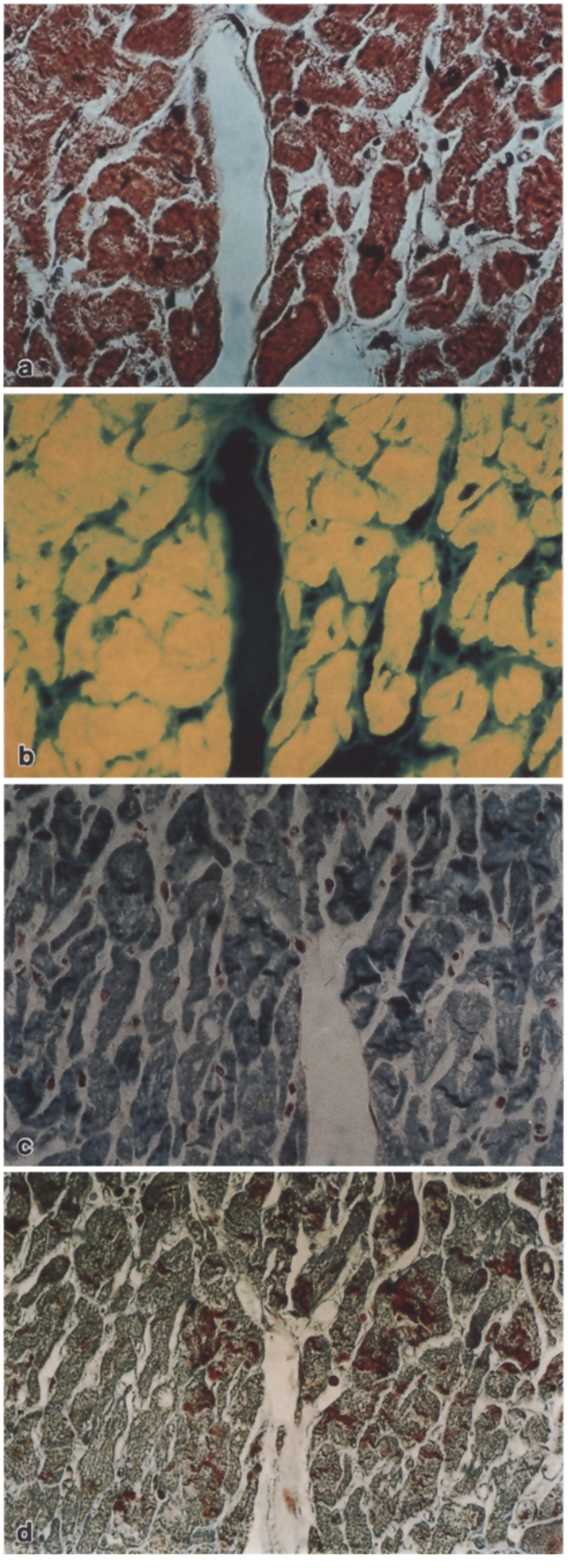

Fig. 1a–d

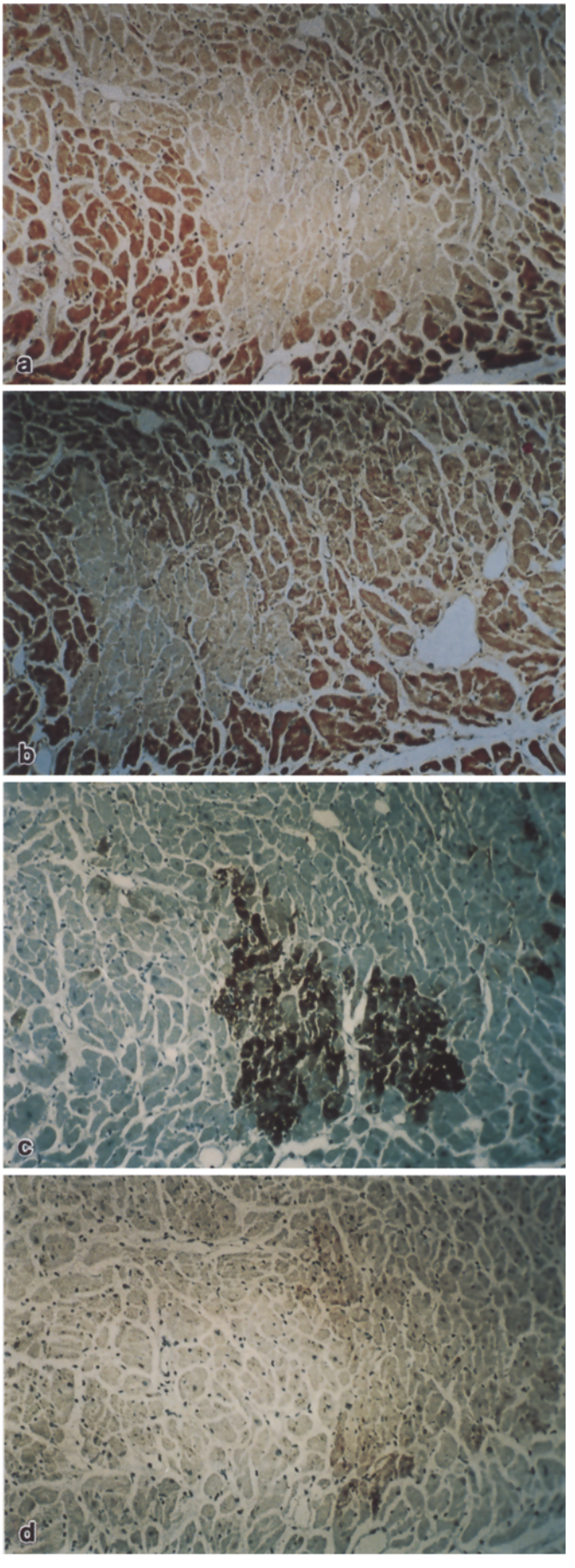

Fig. 2a–d

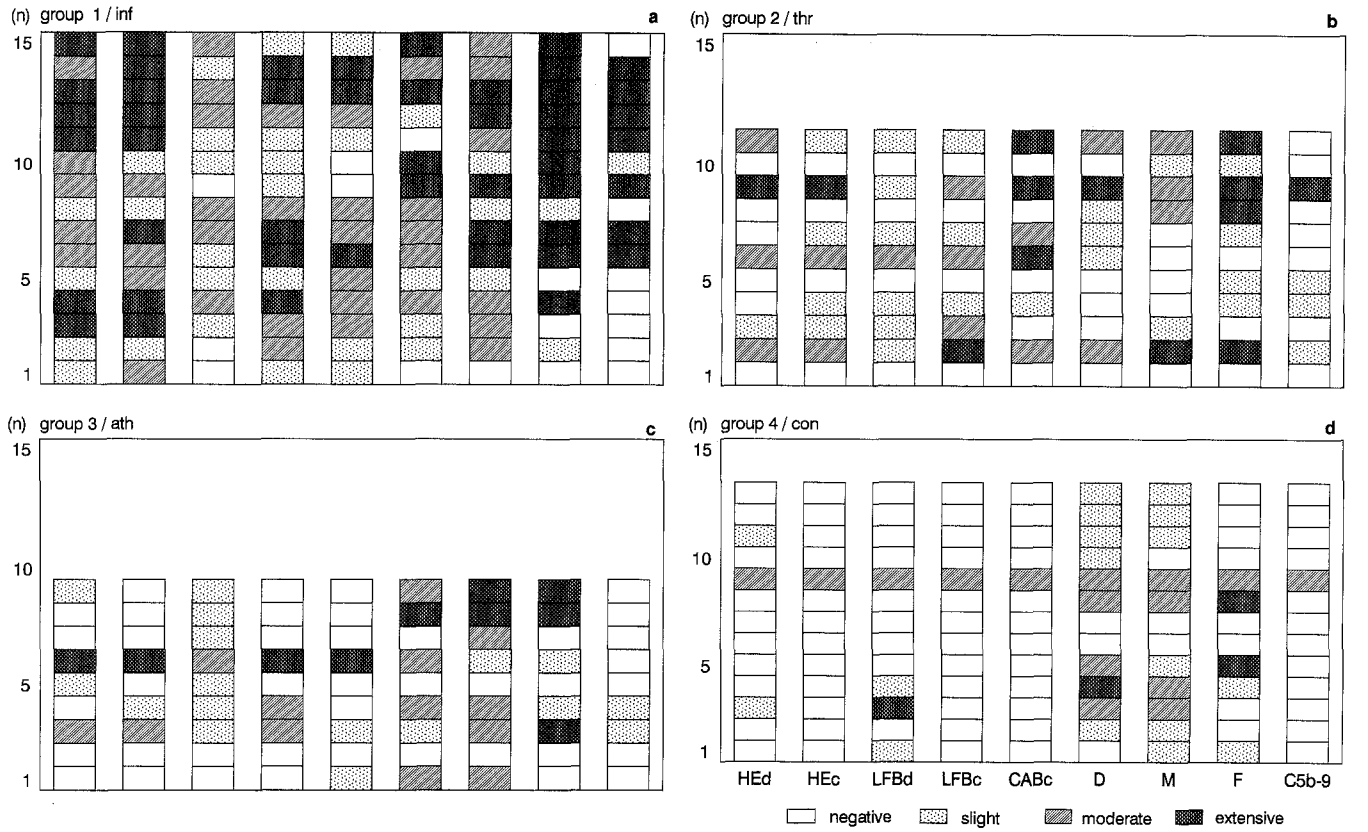

Fig. 3a–d. Types of reactions and reaction strengths of the HIS/IH methods according to the individual cases and arranged into subgroups (see text)

Discussion

We have subgrouped our material into 4 well-defined categories in relation to the type of heart pathology as the cause of death:

(1) The first group (infarction group) showed in general a more intensive expression of the reaction. The HIS and the IH features were fairly evenly distributed. The 2 basic types of ischemic myocardial necrosis, i.e. coagulation necrosis and contraction band necrosis (e.g. Becker & Andersen 1985) were expressed either as the d (= diffuse)-type or as the c (= contraction)-type. Serial sections showed a significant loss of the structural proteins D (= desmin) and M (= myoglobin) and a highly specific accumulation of F (= fibrinogen) and C5b-9 (comp) within the necrotic fibres and/or their intimate surroundings. This pattern therefore substantiates the specificity of the HIS findings.

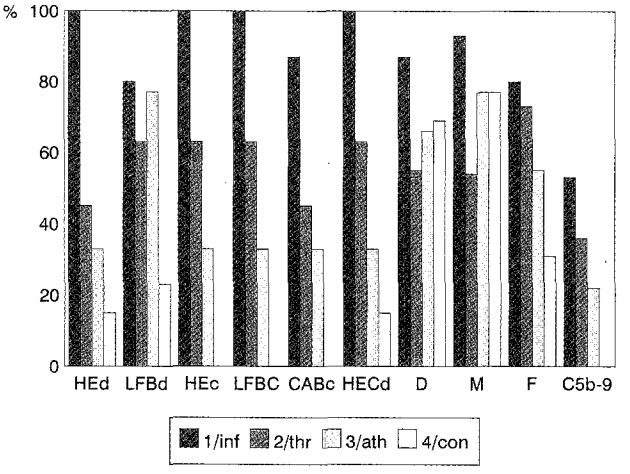

Fig. 4. Frequency of cardiac lesions relative to the HIS/IH methods applied

Fig. 1. Serial sections using different histochemical methods (magnification 460 ×). a. Haematoxylin-eosin (H & E), cardiac muscle fibres with hypereosinophilia, homogeneous cytoplasm (coagulative necrosis and contraction bands (contraction band necrosis). b. H & E-fluorescence, fibres with homogeneous bright yellow fluorescence (HEFd) and contraction bands (HEFc). c. Luxol fast blue (LFD), extensive blue staining of damaged fibres, mainly fibres with contraction bands (LFBc). d. Chromotrope aniline blue (CAB), extensive red-coloured contraction bands (CABc) in the centre

Fig. 2. Serial sections using different immunohistochemical methods (magnification 460×). a. Immunohistochemical detection of desmin (D), homogeneous depletion of desmin in the centre (ischemic fibres), positive reaction in undamaged fibres. b. Immunohistochemical detection of myoglobin (M), different negative reaction in the infarction area, normal fibres with positive reaction. c. Immunohistochemical detection of fibrinogen (F), extensive positive reaction in ischemic area, accumulation of fibrinogen in damaged fibres. d. Immunohistochemical detection of complement C5b-9, slight positive reaction in the infarction area, diffuse accumulation of C5b-9 in single fibres

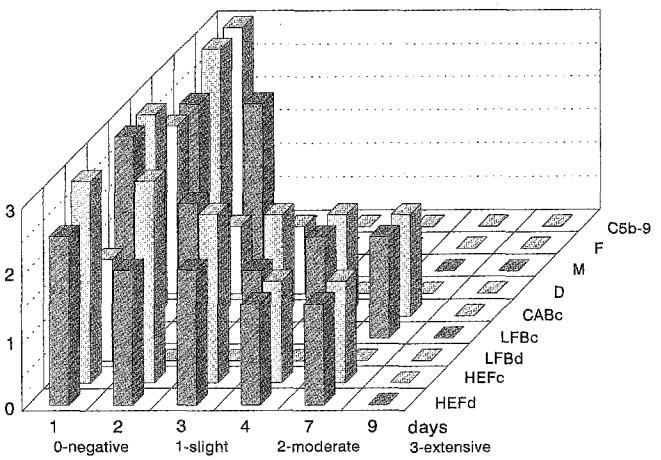

Fig. 5. Experimental autolysis relative to HIS and IH methods

(2) In the second subgroup (thrombosis without infarction) 4 cases out of 11 lacked the histological/histochemical signs of myocardial necrosis and 3 showed "positive reactions" for the antigens tested. Compared to group 1, the mean scores were significantly reduced and only 3 cases exhibited more intensive reaction strengths. This would indicate that death occurred shortly after the coronary occlusion probably due to primary or secondary arrythmia (secondary to infarction) (Hackel and Reimer 1990; Buja and Willerson 1991).

(3) The third subgroup had 4–5 cases (out of 9) with virtually no or only weak signs of myocardial necrosis while 2 reacted negatively with immunohistochemistry. The unequal reduction of the average scores in the HIS markers compared to 3 out of 4 IH markers would further substantiate the interpretation that primary arrythmia led to relatively sudden cardiac arrest either not allowing the manifestation of HIS markers or only their very weak occurrence whilst the markers D, M and F remained mainly unchanged. These seem therefore to develop much faster. In contrast, C5b-9 seems to react even more slowly than the HIS markers. In addition, there was only 1 case with the patchy type of change while the others showed a more or less disseminated single and/or grouped fibre necrosis.

(4) In the fourth group, there was no case with histological/histochemical myocardial necrosis and no case with C5b-9 comp. positivity. The only case with signs of coagulation necrosis (No. 38) was death due to heroin overdose, which can be associated with a long agonal period and would therefore easily explain the manifestation of myocardial alterations. This observation is in contrast to other investigations (Oehmichen et al. 1990a,b) where the myofibrillar type of degeneration was observed even in non-cardiac deaths, but is possibly due to differences with regards to the duration of the agonal periods.

The diagnostic value of the staining procedures

Several investigations have elaborated new histochemical methods and established well-defined types of reactivity. For example using LFB, Arnold (1982, 1985, 1986) introduced the diffuse type of myofibrillar degeneration. In our series, using H&E fluorescence and LFB on serial sections, we came to the conclusion that the diffuse type of lesion is identical in both staining methods. In most of our cases, these 2 signs were positive in parallel but HE seemed to be more. Therefore, the use of LFB for the detection of the diffuse type of myofibrillar degeneration offers no advantages.

Applying the stains H&E, LFB and CAB on consecutive serial sections, we could observe that the terms "contraction band necrosis" (H&E, Kloner et al. 1984), "cross band type myofibrillar degeneration" (LFB, Arnold 1982, 1985, 1986) and "contraction bands" (Zollinger 1983) are only different designations for the same morphological entity. The H&E staining showed 2 more positive cases only in the infarction group which could again indicate enhanced sensitivity of this method. Therefore we are very much convinced that the classical H&E offers the same resolution power if evaluated by light *and* ultraviolet microscopy.

Of the immunohistochemical markers used, D and M reacted very much in parallel and we observed only very few cases where they showed different reactions. In these cases, the myoglobin indicated a higher degree of sensitivity. – The "humoral" markers of early myocardial lesion, namely F and C5b-9 again showed mostly parallel reactions. If the reactions were different, F was positive and C5b-9 negative. This finding, in combination with the decrease of C5b-9 reaction over groups 1–4 would indicate a higher sensitivity of fibrinogen.

We would therefore tentatively recommend that the same diagnostic value could be achieved with a set of 3 methods, i.e. H&E, myoglobin and fibrinogen.

Literature dealing with the diagnostic value of histological and histochemical methods mainly differentiates between sensitivity and specificity which can be misleading if these methods are evaluated in isolation. For example, the specificity of F to detect myocardial infarction can be evaluated as poor if it is based on the reactivity in the control cases. – On the other hand positivity within a fibre specifically indicates necrosis. In a complex physiology such as heart pathology, where the muscle fibres can be primarily or secondarily involved and, where in addition interactions exist between these two possibilities, only the precise evaluation of all features together can be used to really evaluate specificity and sensitivity. The framework could approximately follow this structure:

1) in macroscopical infarction, the methods applied need no evaluation of their sensitivity per se but the reaction pattern relative to topography, survival time and postmortem time and eventual losses and enhancements of the criteria used can serve as tools to answer additional questions such as time dependency.

2) in cases with coronary thrombosis alone, the simultaneous application of all 3 methods can very much enhance both the specificity and the sensitivity of the diagnosis of ischemic myocardial damage and therefore substantially contribute to defining the cause of death.

3) in forensic cases, the pure diagnosis of stenosing coronary atherosclerosis cannot be regarded as the cause of death. This is only possible per exclusionem, e.g. any kind of external influence such as poisoning and/or

by proving acute myocardial damage relative to topography.

4) the positive results found in one control case are not necessarily disadvantageous and only indicate that this can be important for the evaluation of the agonal period and that the presence of myocardial damage alone does not necessarily prove cardiac death.

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