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Immunostaining by Complement C9: A Tool for Early Diagnosis of Myocardial Infarction and Application in Forensic Medicine

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ABSTRACT: Before the first 12 hours, diagnosis of early myocardial infarctions is always difficult for forensic pathologists. We tested complement C9 expression in 121 formalin-fixed and paraffin-embedded heart samples by an immunohistochemical procedure. The heart specimens were separated into four groups: 33 cases in group 1 with typical ischemic damages histologically located, 20 cases in group 2 with death related to myocardial infarction on the basis of ischemic presentation on electrocardiogram but no obvious histological ischemic damage, 35 cases in group 3 with severe coronary disease without cause of death found at the autopsy, and 33 cases in group 4 without sign of myocardial infarction and without coronary disease. In the first group, all 33 heart samples showed a well-defined C9 expression in the necrotic areas. The second group in 17 of 20 cases showed positive areas for C9 expression. In the other three heart specimens, only few stained cells were observed whereas the painful symptoms had begun less than 1 h before death. The third group showed C9 immunopositive areas in six of 35 cases, few stained cells in 8 cases, and no C9 deposition in the 21 other cases. The last group showed no staining area. To avoid nonspecific C9 staining due to tissue autolysis, we studied C9 expression during a controlled putrefactive process in four cases included in group 1; staining was found only in infarcted myocardial areas, and was observed up to ten days. Specificity of C9 expression was evaluated to be 100% [89.4 to 100%] and sensitivity to be 85% [62.11 to 96.79%]. In conclusion, evaluation of immunohistochemical expression of C9 appears to be a highly sensitive and specific marker of early myocardial infarction, useful in forensic medicine if survival is more than 1 h after the beginning of myocyte damage.

KEYWORDS: forensic science, heart, complement, myocardial infarction, immunohistochemistry, early detection, sudden death

Definite diagnosis of sudden or violent death on postmortem samples is always difficult for the forensic surgeon. Cardiovascular diseases come first, representing about 40% of the causes of sudden death (1); half of them are directly related to myocardial infarction or coronary insufficiency (2). The revelation of postmortem features of infarction can be easy in the case of a recognized infarction with well-codified histological findings, but it remains difficult with recent infarctions, less than 12 h old. Over the years, numerous methods of early detection have been suggested: special staining using periodic-acid Schiff, phosphotungstic acid hematoxylin, or hematoxylin basic fuschin picric acid; fluorescence using acridine orange; and biochemical methods for the determination of K+/Na+ ratio, and myoglobin or creatine kinase activity in pericardial fluid (3–6). Their use has today often been abandoned due to their lack of sensitivity or specificity. More recently, immunohistochemical methods have been increasingly used, with antibodies detected against myoglobin, desmin, fibrinogen, troponine, C3b complement, or C5b-9 complement-complex (7–9). C5b-9 was shown to be a reliable marker of very early myocardial tissue injury but these immunocytochemical studies were performed on fresh-frozen autopsy materials, a condition difficult to obtain in forensic practice (8).

Using formalin-fixed and paraffin-embedded tissue sections, we tested the immunohistochemical revelation of the complement C9 in series of infarcted myocardia (10). We used a total of 121 heart samples with either macroscopically defined infarction, documented infarction beginning less than 10 h before death, possible infarction, or no infarction. We compared immunohistological results with histological findings in hematoxylin-eosin-saffran (HES)-stained sections.

Materials and Methods

Human Heart Specimens

We worked on 121 formalin-fixed and paraffin-embedded heart samples taken between 24 and 48 h after death, during the autopsy for scientific purposes. On 72 whole anatomical hearts, we systematically collected the left anterior descending coronary artery, the left circumflex descending coronary artery, the right coronary artery, and two samples of the right ventricle, the septum, the anterior and the posterior walls of the left ventricle, and all suspicious gross lesions. In 49 cases, formalin-fixed and paraffin-embedded samples were taken retrospectively from 1988 to 1998; the same sampling had been carried out. For each of the samples studied, clinical history and cause of death were supplied by hospital records. We analyzed myocardial damages with infarctions at various ages, sudden death with severe atherosclerotic coronary damage, dilated cardiomyopathy without coronary disease, or other causes of death such as neoplasms and infectious diseases.

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We formed four groups. The first group consisted of 33 hearts showing clear myocardial ischemical lesions with the standard technique HES. These cases had a cardiomegalia of 420 g on average (400 to 460) and severe damage of left coronary artery, macroscopically observed.

The second group consisted of 20 hearts with a well-documented history of ischemic myocardial disease, with myocardial infarction less than 10 h old on the basis of ischemic presentation on electrocardiogram, but with no obvious histological features on HES staining. In this second group, 12 hearts with an average weight of 340 g (280 to 360) presented moderate atherosclerotic damages with coronary stenosis from 40 to 50% in left anterior descending coronary artery and left circumflex descending coronary artery and without scar of a previous ischemic accident on gross examination. The other eight hearts with an average weight of 350 g (330 to 370) showed coronary stenosis greater than 80%. In five cases, it involves only the left anterior descending coronary artery, and in three cases both the left anterior descending coronary artery and the left circumflex descending coronary artery, but without scar of a former myocardial infarction. Only two coronary thromboses were observed and related to infarcted areas.

The third group consisted of 35 hearts revealing severe atherosclerotic coronary disease (stenosis greater than 80%) accompanied in 23 cases by lesions of a former myocardial infarction. The cardiomegalia was 420 g on average (400 to 460) and severe coronary disease was observed in seven cases on the left anterior descending coronary artery, both on the left anterior descending coronary artery and on the left circumflex descending coronary artery in 27 cases, and on the three coronary arteries in one case. No feature of recent myocardial infarction was observed on the HES staining. Survival ranged from 1 to 3 h. There was no typical anginous symptomatology and electrocardiogram was normal. The only cause of death suspected in this group was of cardiac origin.

The fourth group consisted of 33 hearts from patients who died of other causes: two of digestive ischemia, 12 of terminal tumoral process, 7 of infectious disease, and 12 of dilated cardiomyopathy. The heart weighed from 280 g to 620 g and showed up to 30% coronary stenosis.

Histological Examination

The samples were fixed for 24 h in 4% neutral buffered formalin, dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin. Serial (5 μm) sections of each sample were cut for HES staining and immunohistochemical procedure for C9. On HES-staining, the histological sections were evaluated by two independent investigators who had no knowledge of the clinical history. The criteria considered as revealing acute infarction were coagulative necrosis, contraction band necrosis, or large edema and hemorrhagic suffusions without reanimation procedures before death.

Immunohistological Analysis

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded tissue sections 5 μm thickness. After deparaffinization in xylene, the slides were then rehydrated in graded alcohols for 2 min each and rinsed in phosphate buffer saline (PBS) pH 7.4 for 5 min. Tissue sections were then heated by pressure cooking using a protocol derived from that of Norton et al. and modified by Morgan et al. (11,12). Slides were boiled in EDTA 1 mM pH 8 buffer during 90 s after boiling-point. Tissues were cooled for 20 min and rinsed in PBS. Tissues were then blocked for 10 min at room temperature with 5% normal goat serum (Dako, Trappes, France) in PBS with 0.5% nonfat dry milk (PBSM). Excess blocking solution was drained and replaced with a polyclonal goat anti-human C9 antibody (The Binding-Site, Birmingham, UK) diluted at a working solution of 1:5000 in PBSM with 5% normal goat serum and incubated on tissue sections for 60 min at room temperature. Slides were washed by two brief PBS rinses and one 5 min immersion in PBS. Subsequently, slides were incubated for 30 min at room temperature in a 1:5000 dilution of peroxidase-labeled streptavidin (Dako). The slides were again washed in PBS and incubated 10 min in amino-ethylcarbazole as peroxidase substrate. The unreacted chromogen was removed by immersion in running tap water, and the slides were counterstained with Mayer's hematoxylin for 10 min. They were mounted with glycergel (Dako). For each heart, identical tissue sections were prepared for negative controls by substituting immune serum by normal goat serum.

The immunostaining pattern was evaluated by the same investigators according to the size of myocardial infarction: 0 = no C9 deposition observed; + = immunostaining of disseminated single cells or few small piles of cells; ++ = immunostaining of large areas of infarcted cells. Moreover, to avoid nonspecific C9 staining due to tissue autolysis, we took a sample of infarcted myocardial tissue and a sample of nonischemic area every day in four cases of group 1, in order to study modifications of the immunostaining during a controlled putrefactive process. The samples were kept in a nonsterile closed jar at a constant temperature of 25°C.

Statistical Analysis

Sensitivity and specificity were estimated with exact confidence interval using control group 4 and groups 1 and 2 (13).

Results

Immunohistological staining using polyclonal antibody to C9 was attempted to demonstrate ischemic necrotic areas. Table 1 shows immunohistochemical results, and Table 2 shows the statistical analysis.

TABLE 1—*Immunostaining pattern of the four groups.*

	Number of Cases	C9 = 0	C9 = +	C9 = ++
Group 1	33	0	0	33
Group 2	20	0	3	17
Group 3	35	21	8	6
Group 4	33	33	0	0

NOTE: The immunostaining was evaluated according to the size of myocardial infarction, with the following criteria: 0 = no C9 deposition observed; + = immunostaining of disseminated single cells or few small piles of cells; ++ = immunostaining of large areas of infarcted cells.

TABLE 2—*Specificity and sensitivity of C9 expression.*

	Positive Result: C9 = ++
Group of cases (*n*)	Sensitivity [95% CI]
Group 1 (33)	100% [89.42% - 100%]
Group 2 (20)	85% [62.11 - 96.79]
Group 1 + group 2 (53)	94.34% [84.34 - 98.82%]
Group 4 (33)	Specificity 100% [89.4% - 100%]

NOTE: C9 = 0 or C9 = + was considered a negative result. The sensitivity could be estimated from group 1, or group 2, or groups 1 and 2 pooled. If C9 immunohistological procedure is done only in cases HES staining was negative, the sensitivity would be that for group 2.

In the first group, the 33 hearts tested revealed intense immunostaining that formed a patchwork including areas where all the myocardial cells were labeled next to areas totally unstained. The immunostaining was cytoplasmic and sometimes membranous. These immunohistologically stained areas coincided with the ischemic lesions found on HES sections (Figs. 1 and 2). In all the cases, the borders of these various areas were clearly defined, abrupt, and with no transitional zone; they were more strongly immunostained on the outskirts of the infarcted areas (Figs. 3 and 4).

FIG. 1—*Myocardial infarction after 18 h surviving: ischemical lesions of coagulative necrosis in HES section (Original magnification × 100).*

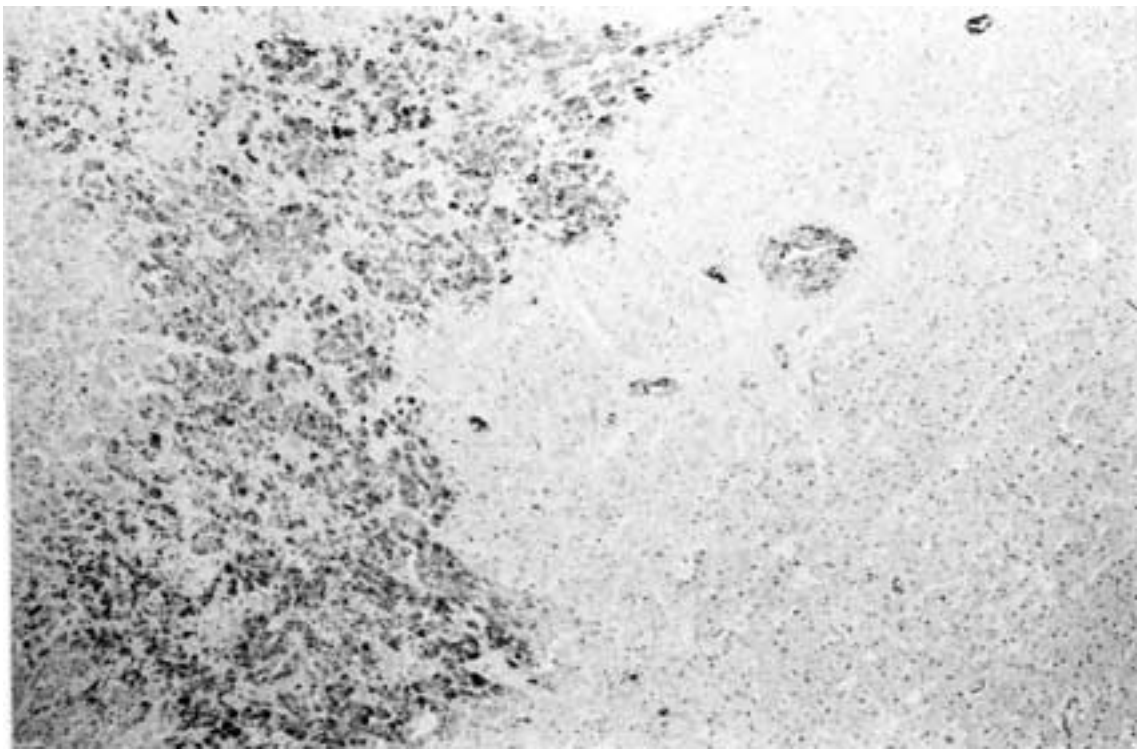

FIG. 2—*Immunostaining coincided with the ischemic damage. Internal control was strongly present (Original magnification × 50).*

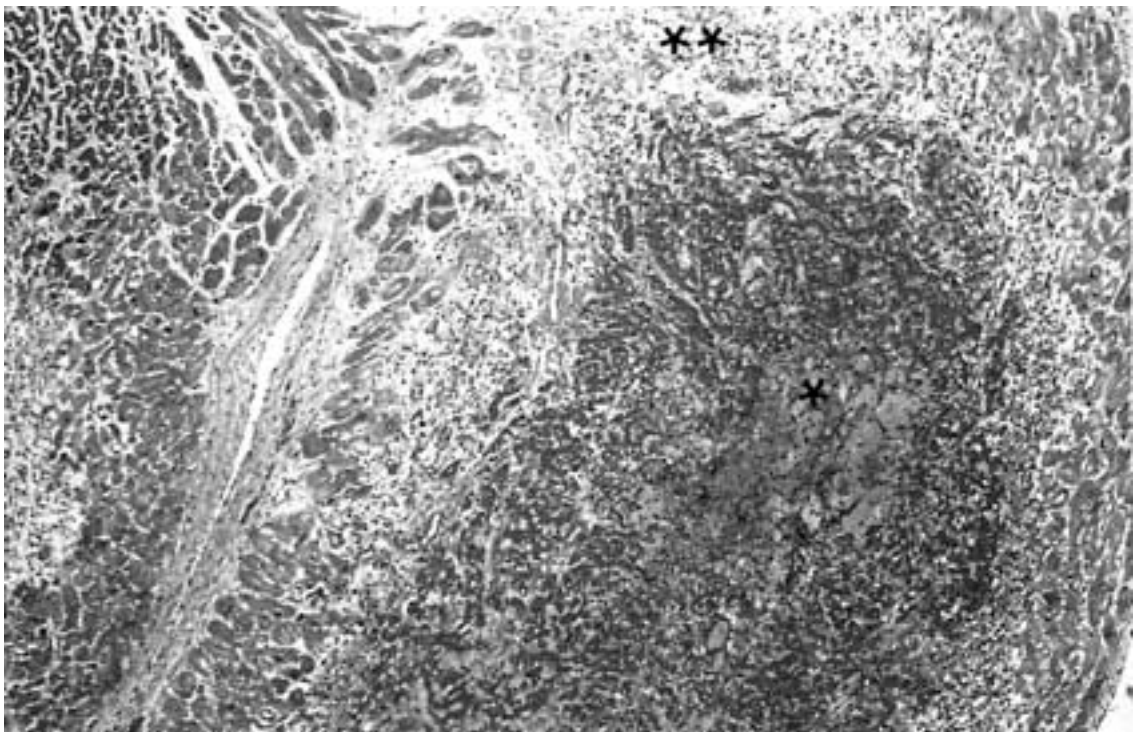

FIG. 3—*Myocardial infarction after 24 h surviving: ischemic lesions with cellular hypereosinophilia, edema, and hemorrhagic suffusions (*) surrounded by older infarction in HES section (**) (Original magnification × 100).*

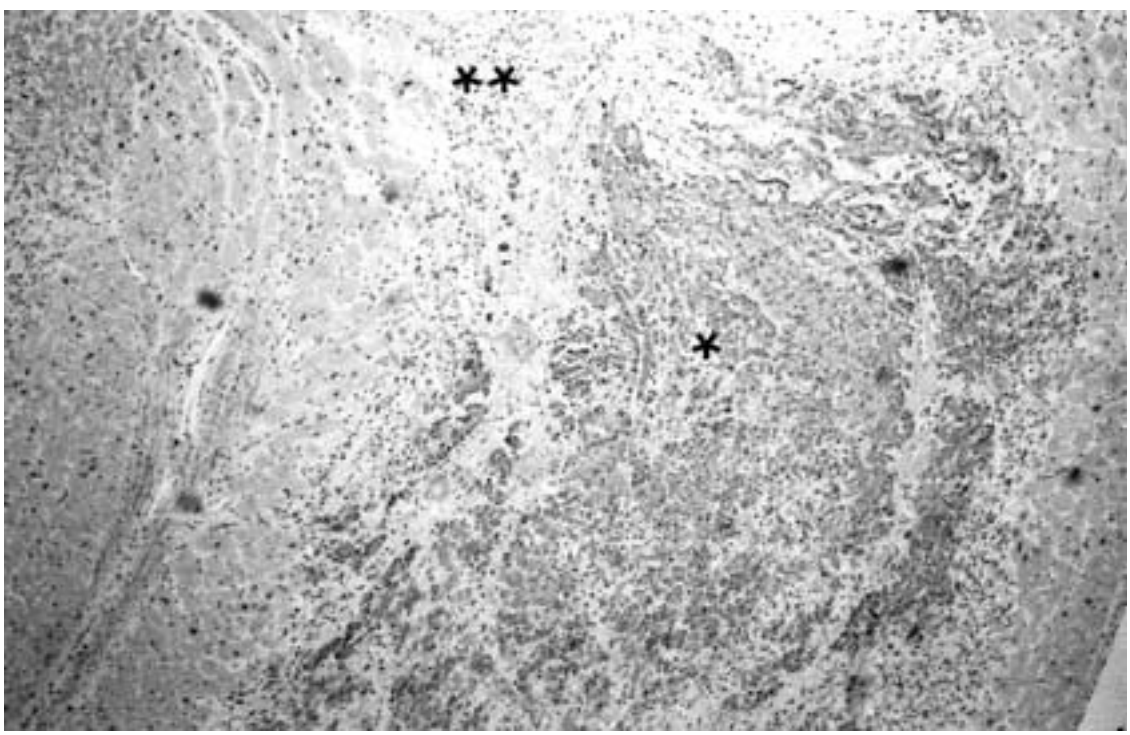

FIG. 4—*Immunostaining coincided with recent ischemical damage but the staining did not involve the older lesion (Original magnification × 50).*

The 20 cases of the second group showed group two patterns of immunostaining. The first subgroup included 17 cases with intense and large immunostained areas, as in group 1. By contrast their lesions of cellular edema and cytoplasmic hypereosinophilia observed on HES staining sections were mild (Figs. 5 and 6). These 17 cases corresponded to different timescales in the evolution of the pathological process: death occurred between 30 min and 2 h in four cases, 3 and 6 h in four cases, and 7 and 12 h in nine cases after the beginning of the painful symptoms corresponding to ischemic myocardial process. In the other subgroup (three cases),

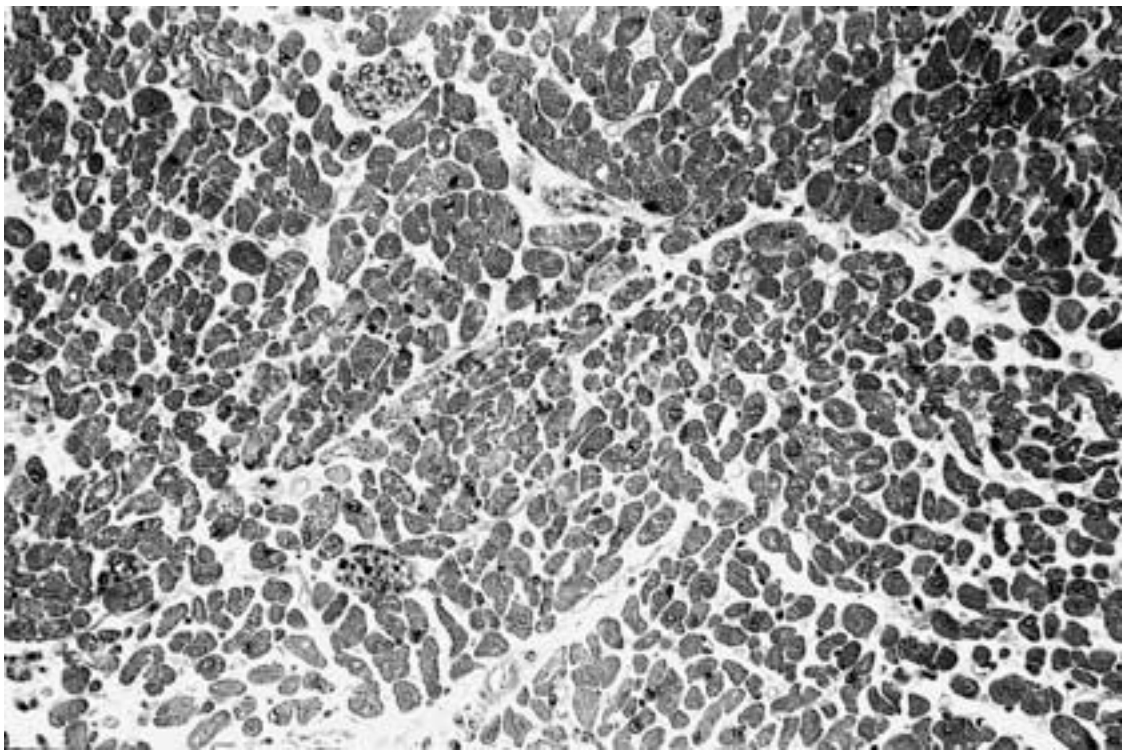

FIG. 5—*No ischemic lesion was visible in HES section, only irregular cellular shape in cardiomegalia. Death only 2 h after ischemic pain began (Original magnification × 200).*

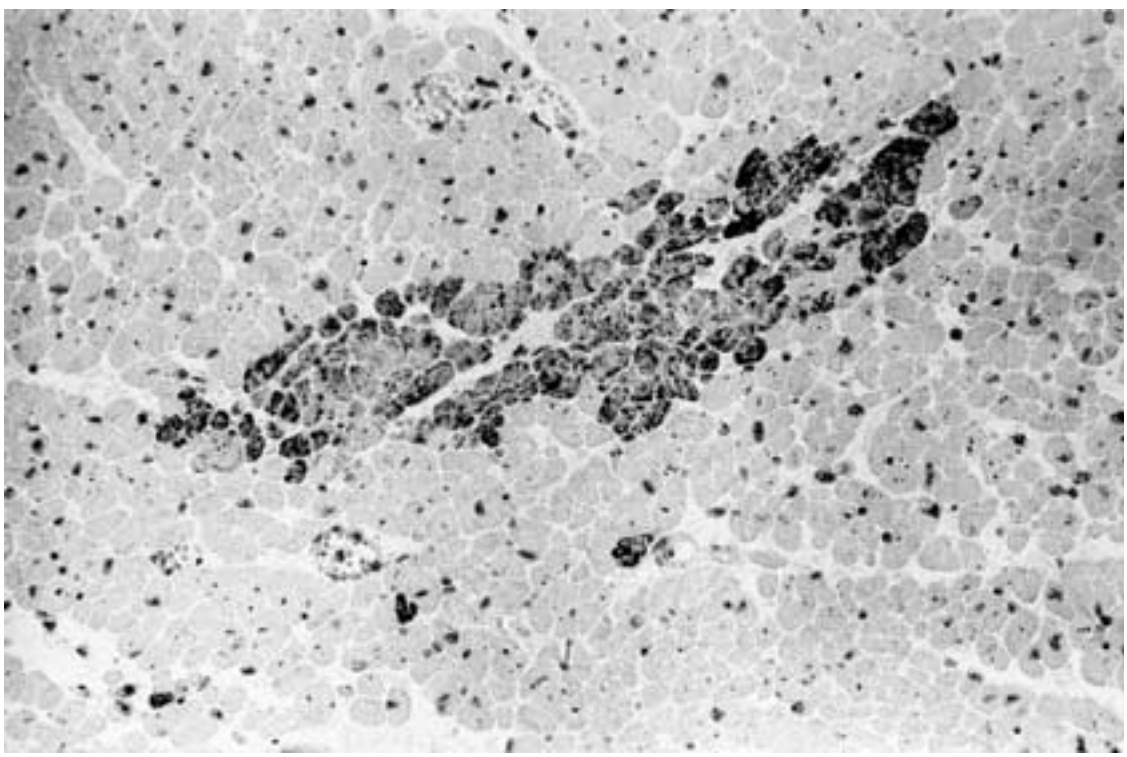

FIG. 6—*Ischemic cells foci well identified by immunohistochemical procedure (Original magnification × 200).*

ischemic lesions were revealed by immunostaining on few cellular elements, isolated or in small groups. This was considered insufficient damage to be the cause of death. Note that in this group, the painful symptoms began less than 1 h before death (15 min in one case, and 30 min in two cases).

In the 35 heart samples of the third group, 14 showed immunostaining, from a large systematized area in six cases to a few cells, isolated or in small piles, in eight cases.

In the 33 heart specimens of the fourth group which were taken on patients who died from an extra-cardiac cause or on the hearts explanted for dilated cardiomyopathy with no coronary disease, no immunostained area was noticed.

The analysis of the influence of putrefaction showed an intense and specific staining present up to ten days only on the infarcted areas after the experimental autolysis had begun. Staining intensity then decreased and disappeared after 15 days. On the other hand, no immunostaining was observed in healthy areas.

An internal staining control was observed; it affected the arteries whose subendothelial layers showed atherosclerotic injury (Fig. 2). Vascular complement deposition appeared to correlate with the age of the patient and the degree of atherosclerosis (14). It was observed in the putrefactive controlled process up to 15 days.

Discussion

The hypothesis that the complement played a role in myocardial damages was first made in 1971 by Hill and Ward (15). These authors showed that the injured myocardial tissue released a protease responsible for the cleavage of fraction C3. Depletion in C3 before ischemia, for example, by administering soluble CR1, an endogeneous inhibitor of the complement, prevented the penetration of the neutrophils in the ischemic tissue. Fraction C3 is found after 4 h of ischemia in the myocardial cells of the baboon. It is confined to myocyte membranes and, after 24 h of evolution, on vascular walls (16). The plasmatic levels of the circulating fragments C1, C2, C3, and C4 dropped significantly among patients 24 to 48 h after the ischemical process began, evoking an increased consumption (17). The activation of the complement with a release of biologically active fragments then contributed to worsening in the infarcted areas. Indeed, direct tissue damage is mediated by membrane attack complex (C5b-9) and possibly by direct vasoconstriction properties of C5a (18). Insertion of the C5b-9 complex into a myocyte or endothelial cell membrane may lead directly to cell necrosis, whereas vasoconstriction exacerbates ischemia. Using the immunohistochemical procedure on frozen sections, Schäefer et al. identified other factors of the complement, from the C5 to the C9 fractions (19), whereas these components were not detected on healthy myocardium (20).

The cause of complement activation in ischemic myocardial areas remains unknown. A working hypothesis is that cellular ischemia results in loss of the myocyte membrane ability to inactivate spontaneous deposited C3b, leading directly to C5 and C5b-9 activation (21). Generation of hydrophilic channels would permit spontaneous rapid influx of calcium inside the myocyte. The deleterious effects of high intracellular calcium concentration are well established (22). The loss of myocyte integrity and the resulting release of creatinine kinase suggest complement activation in the interstitial fluid at the myocyte surface. This suggestion is supported by the appearance of the soluble C5b-9 macromolecular complex in the lymphatic fluid at 30 min. Yasuda et al. reported similar findings and demonstrated a correlation between the soluble C5b-9 concentration and peak plasma creatine kinase concentration (23). Homeister demonstrated that complement activation can cause myocardial tissue damage in the absence of neutrophil recruitment and infiltration (18).

The use of frozen material cannot be adapted to the daily practice of forensic medicine. Freezing means that only few and small samples could be taken. Moreover, when the judicial police officers put the seals on, an appropriate container for freezing must be used so that these samples can be stored for a second assessment. Inversely, revealing C9 by immunohistochemistry, has five advantages. It is an easy and reproductible method that does not require any special and expensive material or reagents. Paraffin-embedded specimens allow satisfactory retrospective study because the staining remains over time: fragments ten years old remain intensely stained on. Second, the procedure based on formalin-fixed tissue section does not alter forensic surgeon habits and does not hinder their autopsy observations. It seems essential, however, to take several myocardium sections, especially septum, right, and left ventricle specimens during any forensic autopsy for unexplained deaths. Third, the procedure is very sensitive, so expression of an isolated necrotic cell may be detected in a myocardic fragment showing no immunostaining whatsoever. Complement C9 immunostaining revelations were superimposable on ischemic injuries observed in HES staining in group 1, with an heterogeneous immunostaining forming a patchwork with areas of unstained cells. Fourth, the nonischemic myocardium never expresses complement C9. Note that Rus et al. found a very small quantity of complement in the normal myocardial tissue with the ELISA method but not by immunohistochemistry (24). Normal cells do not activate the complement system and are fairly resistant to autolysis despite the presence of C5b-9 (24). Fifth, the antigenic expression is specific and nonartifactual by physiological autolysis. The absence of these deposits in the healthy areas or around the infarcted areas without histological lesions excludes the possibility of a complement activation secondary to the death as a consequence of cellular alterations linked with the putrefaction. In paraffin-embedded samples, no false positives were observed after experiment, as described by Thomsen and Held for frozen sections (25). Moreover, C9 deposition was never observed in other causes of deaths (group 4), as reported by Thomsen and Held (26). Also, we found no staining of cellular lesions due to cardiac massages or any direct trauma with lesion visible macroscopically; these results are similar to those obtained by Fechner et al. with C5b-9 on frozen material (27). In these cases, Thomsen and Held suggested that the time of survival is too short (< 30 min) to lead to the formation of the membrane attack complex (26).

C9 complement immunostaining is helpful for the detection of myocardial infarction when the survival is less than 12 h with no histological feature on HES staining. Complement C9 deposition is clearly observed as soon as 1 h and is always present with intense and large stained areas if survival goes beyond 3, as we observed in group 2. One of the 3 cases of group 2 with a survival of 30 min showed discordant immunostaining results; in this case staining areas were large and intense membranous immunostaining was present, suggesting the first phase of the C9 deposit.

In group 3, to the extent that statistical analysis showed a highly specific and sensitive complement C9 expression, and if C9 = + is not considered a positive result because only small infarcted areas were stained, there were only six cases of myocardial infarction. So, another cause of death is possible. Autopsy and toxicologic examination must be revised. Nonetheless, heart failure without myocardial infarction is still conceivable.

Evaluation of immunohistochemical expression of C9 appears to be a highly sensitive and specific marker of early myocardial infarction, useful in forensic medecine if survival is more than 1 h after the beginning of myocyte damage.

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References

1. Knight B. The pathology of sudden death. Second Edition In: Forensic Pathology Ed Arnold London 1997:487–503.
2. Brya LM, Willerson JT. Relationship of ischemic heart disease to sudden death. J Forensic Sci 1991;36:25–33.
3. Knight B. Investigation of sudden deaths from myocardial ischaemia. Forensic Sci Int 1976;8:33–6.
4. Knight B. A further evaluation of reliability of the HBFP stain in demonstrating myocardial damage. Forensic Sci Int 1979;13:179–81.
5. Leadbeatter S, Wawman HM, Jasani B. Immunocytochemical diagnosis of early myocardial ischaemic/hypoxic damage. Forensic Sci Int 1989;40:171–80.
6. Hougen HP, Valenzuela A, Lachica E, Villanueva E. Sudden cardiac death: a comparative study of morphological, histochemical and biochemical methods. Forensic Sci Int 1992;52:161–9.
7. Leadbeatter S, Wawman HM, Jasani B. Further evaluation of immunocytochemical staining in the diagnosis of early myocardial ischaemic/hypoxic damage. Forensic Sci Int 1990;45:135–41.
8. Brinkmann B, Sepulchre MA, Fechner G. The application of selected histochemical and immunohistochemical markers and procedures to the diagnosis of early myocardial damage. Int J Leg Med 1993;106:135–41.
9. Hansen SH, Rossen K. Evaluation of cardiac troponin I immunoreaction in autopsy hearts: a possible marker of early myocardial infarction. Forensic Sci Int 1999;99:189–96.
10. Doran JP, Howie AJ, Townend JN, Bonser RS. Detection of myocardial infarction by immunohistological staining for C9 on formalin fixed, paraffin wax embedded sections. J Clin Pathol 1996;49:34–7.
11. Norton AJ, Jordan S, Yeomas P. Brief high temperature heat denaturation (pressure cooking): a simple and effective method of antigen retrieval for routinely processed tissues. J Pathol 1994, 173:371–9.
12. Morgan MM, Nanabi H, Schmidt K, Janasi B. Possible role of tissue-bound calcium ions in citrate-mediated high temperature antigen retrieval. J Pathol 1994, 174:301–7.
13. Laplanche A, Com-Nougué C, Flamant R. Méthodes statistiques appliquées à la recherche clinique. Ed Flammarion Médecine Science 1987.
14. Vlaicu R, Niculescu F, Rus HG, Cristea A. Immunohistochemical localization of the terminal C5b-9 complement complex in human aortic fibrosis plaque. Atherosclerosis 1985;57:163–77.
15. Hill JH, Ward PA. The phlogistic role of C3 leukotactic fragments in myocardial infarcts of rats. J Exp Med 1971;133:885–900.
16. Pinckard RN, O'Rowke RA, Crawford MH, et al. Complement localization and mediation of ischemic injury in baboon myocardium. J Clin Invest 1980;66:1050–6.
17. Pinckard RN, Olson MS, Giglas PC, Boyer JR, O'Rowke RA. Consumption of classical complement components by heart subcellular membranes in vitro and in patients after acute myocardial infarction. J Clin Invest 1975;56:740–50.
18. Homeister JW, Satoh P, Lucchesi BR. Effects of complement activation in the isolated heart; Role of the terminal complement. Circulation Research 1992;71:303–19.
19. Schäfer H, Mathey D, Hugo F, Bhakdi S. Deposition of the terminal C5b-9 complement complex in infarcted areas of human myocardium. J Immunol 1986;137:1945–9.
20. Crawford MH, Grover FL, Kolbs WP, McMahan RA, O'Rourke LM. Complement and neutrophil activation in the pathogenesis of ischemic myocardial injury. Circulation 1988;78:1449–58.
21. Vakeva A, Morgan BP, Tikkanen F, Helin K, Laurila P, Meri S. Time course of complement activation and inhibitor expression after ischemic injury of rat myocardium. Am J Pathol 1994;14(6):1357–68.
22. Mathey D, Schofer J, Schäfer HJ, et al. Early accumulation of the terminal complement-complex in the ischaemic myocardium after reperfusion. Eur Heart J 1994;15:418–23.
23. Yasuda M, Takeuchi K, Hiruma M, et al. The complement system in ischemic heart disease. Circulation 1990;81:156–63.
24. Rus HG, Niculescu F, Vlaicu R. Presence of C5b-9 complement complex and S-protein in human myocardial areas with necrosis and sclerosis. Immunol Lett 1987;16:15–20.
25. Thomsen H, Held H. Susceptibility of C5b-9 to postmortem changes. Int J Leg Med 1994;106:291–3.
26. Thomsen H, Held H. Immunohistochemical detection of C5b-9 in myocardium: an aid in distinguishing infarction-induced ischemic heart muscle necrosis from other forms of lethal myocardial injury. Forensic Sci Int 1995;71:87–95.
27. Fechner G, Bajanowski TH, Brinkmann B. Immunohistochemical alterations after muscle trauma. Int J Leg Med 1993;105:203–7.

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