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ORIGINAL ARTICLE

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Immunohistochemical detection of early myocardial infarction. An evaluation of antibodies against the terminal complement complex (C5b-9)

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Abstract Antibodies (abs) against the terminal complement complex (C5b-9) were used on routinely processed post mortem myocardial tissue in parallel with conventional staining methods. Both monoclonal and polyclonal abs were tested using the avidin biotin peroxidase complex (ABC), alkaline phosphatase anti-alkaline phosphatase (APAAP) methods and an ab-bridge with alkaline phosphatase. Enhancement of the diaminobenzene (DAB) end product with cobalt-nickel (ABC method) was also done. The polyclonal ab gave the most satisfactory results and the alkaline phosphate conjugated ab-bridge had a slight advantage over the ABC method. Cobalt-nickel enhancement of DAB improved the visualization, but with higher background staining. APAAP was the least satisfactory method. Comparing the immunohistochemical method with the conventional staining methods, the former showed positive reaction in 97% of areas of coagulation necrosis and in 65% of contraction band necrosis. On the other hand coagulation necrosis was seen in 44% and contraction band necrosis in 68% of C5b-9 positive areas indicating that C5b-9 abs react with ischemically damaged myocytes before visible alterations are seen in hematoxilin-eosin staining. Moreover, using C5b-9 abs, it seems possible to exclude agonal/artefactual contraction bands which show a negative reaction. Immunohistochemical detection of C5b-9, using an adequate technique could increase the possibility to demonstrate early ischemic myocardial damage.

Key words Myocardial infarction · Complement membrane attack complex · Immunohistochemistry Sudden Death

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Introduction

Immunohistochemical methodology in the diagnosis of myocardial infarction and early myocardial ischemia may have important applications in forensic pathology. Leadbetter et al. (1989) tested a variety of antibodies (abs) against intra- and extracellular molecules, which were known to be present or depleted in ischemically damaged myocardium. They found that abs against myoglobin, myosin, ceruloplasmin, prealbumin and C-reactive protein, gave the most reliable results. Brinkmann et al. (1993) have shown that with hematoxylin-eosin staining in combination with immunohistochemical methods, i.e. myoglobin, fibrinogen and C5b-9 ab, the accuracy of diagnosis in cases of coronary artery disease and suspect myocardial infarction will be increased. However, in early ischemic injury many abs were found not to discriminate with certainty between true ischemic alterations and purely agonal changes (Leadbetter et al. 1990).

The C5b-9 ab is thought to be specific in necrosis and to be rather insensitive to autolysis (Bhakdi and Tranum-Jensen 1983; Brinkmann et al. 1993). Schäfer et al. (1986), Hugo et al. (1990), and Thomsen et al. (1990) have shown that C5b-9 accumulates in ischemic areas of the myocardium. But there have been some technical problems with monoclonal abs especially on formalin-fixed and paraffin-embedded material (Schäfer et al. 1986; Thomsen et al. 1990). Several new poly- and monoclonal abs are now commercially available. In this study we have used one polyclonal (Calbiochem) and one monoclonal (Dakopatts) ab and compared different immunohistochemical methods. The immunohistochemical method was then compared with the hematoxylin-eosin and Mallory's phosphotungstic acid hematoxylin (PTAH) staining methods in cases of sudden cardiac death with and without coronary artery disease.

Materials and methods

The study is based on samples from 81 autopsies performed between 1991 and 1993 at the Department of Forensic Medicine in Stockholm comprising 65 males and 16 females. The mean age was 45.8 ± 18.9 (SD) years (y) (range 17–86 y). The cases studied could be divided into 3 groups: sudden death with coronary artery disease (n = 44), sudden unexplained death without coronary artery disease (n = 25) and controls (suicidal hangings without coronary artery disease) (n = 12).

From each autopsy 5 tissue blocks were taken from the circumpherence of a transversal section of the heart halfway between the valvular plane and the apex including the septum, anterior, lateral and posterior parts of the left ventricle and one block from the posterior wall of the right ventricle. Tissues were fixed in a 4% phosphate buffered formalin solution and processed routinely. The period of fixation varied between 1 and 3 d. Paraffin sections of 4 µ thickness were placed on poly-l-lysine covered slides for immunohistochemistry and parallel sections on untreated slides for routine staining with hematoxylin-eosin and PTAH.

The following immunohistochemical methods for visualization of the antibody-antigen reaction were used: the avidin biotin peroxidase complex (ABC) and the alkaline phosphatase anti-alkaline phosphatase (APAAP) methods. We also used an indirect method employing an ab-bridge conjugated with alkaline phosphatase (AP). With the ABC method we tried to improve visualization by enhancement of the diaminobenzene (DAB) reaction end product with cobalt and nickel (Co-Ni) (Adams 1981). For immunohistochemistry sections were rinsed in a Tris saline buffer at pH 7.6 and treated with pronase (SIGMA). Both the mono- and polyclonal abs were diluted 1:25 in normal rabbit serum (ABC and APAAP methods) or goat serum (AP method) and incubated on the slides overnight at +4° C and an additional 1½ h at room temperature. The slides with polyclonal ab were treated with swine anti-rabbit IgG ab at a 1:300 dilution in 1% normal swine serum (ABC and APAAP methods) or with a bridge goat anti-rabbit IgG ab, conjugated with alkaline phosphatase (Dakopatts, D487), diluted 1:25 in 1% normal goat serum (AP method). The incubation time was 30 min in all cases. After rinsing in Tris saline buffer, ABC-kit solution (Dakopatts) and DAB (SIGMA), or APAAP-kit solution (Vector 1) and alkaline phosphatase substrate chromogen (Vector red) were applied. Enhancement of the DAB reaction product was performed in selected cases in a 1% cobalt chloride/4% nickel ammonium sulphate solution (Adams 1981). A total of 405 slides with 81 negative controls (without C5b-9 ab but otherwise identically treated) and 81 positive controls was made for every individual case. Each slide was studied blindly, once by both authors and then re-evaluated after several months. Comparisons were made between 2 sets of data and expressed in percentages of the total.

Results

Monoclonal ab against C5b-9 generally showed a weaker reaction than the polyclonal ab. With enhancement of the DAB reaction product with cobalt-nickel, a satisfactory result could be reached even with monoclonal ab. The polyclonal ab in combination with the ABC method resulted in good visualization of C5b-9, and the ABC method with cobalt-nickel enhancement increased contrast. With the AP method, giving a red coloration of infarcted areas, the result was at least equally good (Fig. 1). The APAAP method was not satisfactory with monoclonal or polyclonal abs.

In every section an inbuilt positive control was found in the form of C5b-9 positive material in the walls of blood vessels and in mast cell granules. Artefacts were often seen and with experience easy to eliminate, mainly tiny spots that did not follow the outline of the myocytes and filamentous deposits near the edges of the sections.

In the study of separate lesions in the 405 slides from 81 hearts, polyclonal C5b-9 ab combined with the AP

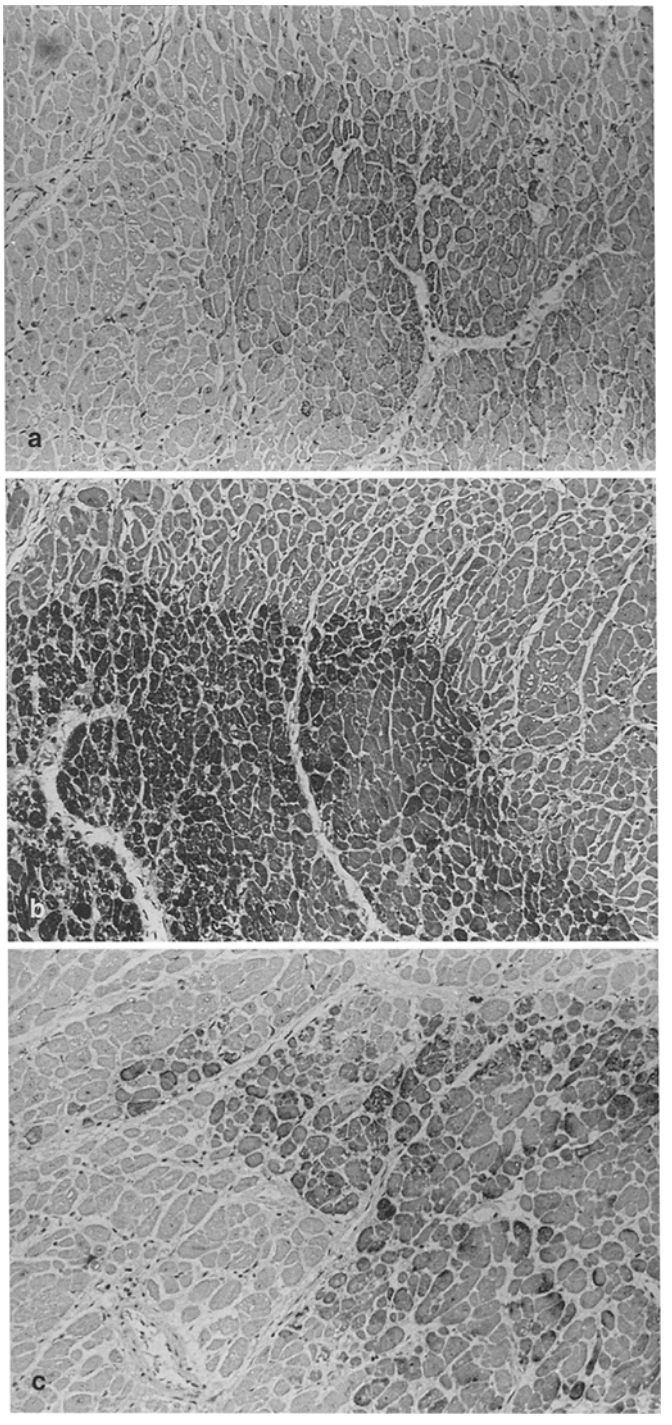

Fig. 1 a–c Myocardial infarction visualized by polyclonal antibody to the terminal complement complex (C5b-9), using **a** the avidin biotin peroxidase complex (ABC) method, **b** intensification of the DAB reaction product with cobalt-nickel, and **c** alkaline-phosphatase conjugated antibody bridge

Table 1 Sensitivity of the immunohistochemical method for demonstration of C5b-9 in myocardial lesions, CN = coagulation necrosis, CB = contraction band necrosis, C5b-9pos = positive reaction in parallel sections. The total number of sections studied was 405

Lesion	*n*	C5b-9 positive (*n*)	Sensitivity %
CN	78	76	97
CB	170	111	65
C5b-9	171	171	100

Table 2 "Specificity" of the immunohistochemical method for demonstration of C5b-9 in myocardial lesions. CNpos = coagulation necrosis found in parallel sections from C5b-9 positive areas. CBpos = contraction band necrosis found in parallel sections from C5b-9 positive areas, expressed as percentage of the number of C5b-9 positive sections

Lesion	*n*	CN positive %	CB positive %
C5b-9	171	44	68

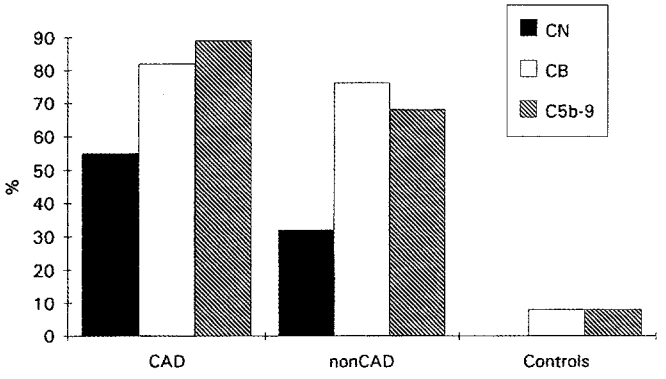

Fig. 2 Coagulation necrosis (CN), contraction band necrosis (CB) and C5b-9 positive areas of myocardium in 81 autopsies, 44 sudden deaths with coronary artery disease (CAD) and 25 unexplained deaths with no coronary lesions (nonCAD) and 12 controls (suicidal hangings with no coronary disease). Y-axis shows the percentage of the total number of cases in each group which showed CN, CB or C5b-9 positive lesions in one or more sections

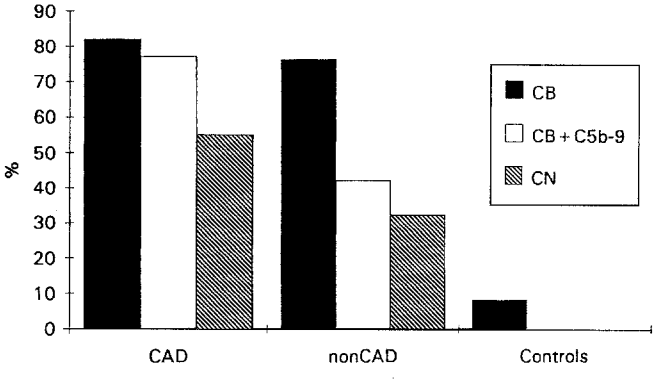

Fig. 3 Subtraction of C5b-9 negative CB lesions (agonal CB) from the total number of CB compared with cases (%) in each group showing coagulation necrosis (CN) in myocardial sections from 81 autopsies, 44 sudden deaths with coronary artery disease (CAD), 25 cases of unexplained deaths lacking coronary lesions (nonCAD) and 12 controls (suicidal hangings with no coronary disease). Y-axis shows the percentage of the total number of cases in each group

method was compared with hematoxylin-eosin and PTAH staining methods. The lesions studied were (i) coagulation necrosis (CN) defined as areas with eosinophilia, nuclear abnormalities, loss of cross-striation in the myocytes and infiltration of neutrophil granulocytes, (ii) contraction band necrosis (CB), i.e. areas with loss of cross-striation of the myocytes and accumulation of irregular hyperchromatic intracellular bands (Baroldi et al. 1979), elucidated by the PTAH stain. In the 405 myocardial section, 171 were C5b-9 positive, 170 showed CB and 78 CN. Out of the 78 CN lesions 76 (97%) were positive with the C5b-9 ab, whereas 111 of the 170 CB lesions (65%) were positive. This was defined as the sensitivity of the C5b-9 ab, with the method used (Table 1). Conversely, in the 171 slides with positive C5b-9 lesions, CN were seen in 76 (44%) and CB in 116 (68%), which was defined as the "specificity" of the C5b-9 ab (Table 2).

From each of 81 patients included in the study 5 areas from the heart were studied. Of these patients 44 belonged to the group with severe CAD. Of these 24 (55%) showed recent myocardial infarction in the form of CN in one or more of the 5 areas. In 36 (82%) patients CB were found and in 39 (89%) individuals C5b-9 positive lesions were seen. The group of sudden death without CAD included 25 cases out of which 8 (32%) had CN in one or more areas, 19 (76%) CB and 17 (68%) displayed C5b-9 positive necrosis. Among the controls CB were seen in 1 case (8%) and C5b-9 positive myocytes, which could not be disregarded as an artefact in an other (8%) (Fig. 2). Contraction band lesions that were not C5b-9 positive were regarded as agonal or artefactual. The number of cases with combined CB and C5b-9 positive lesions exceeded that of CN alone in both the CAD and non-CAD groups, 77% vs 55% and 42% vs 32% respectively and the CB in the controls were found to be agonal (Fig. 3).

Discussion

C5b-9 was chosen as a marker for myocardial infarction because it accumulates specifically on the surface of necrotic cells and might also be the final cause of the damage by opening pores on the cellular membrane (Bhakdi and Tranum-Jensen 1983). Irreversible ischemic damage, has been shown to start as early as 20 min after total occlusion of a coronary artery (Kloner et al. 1974). It would thus be theoretically possible to detect the terminal complement complexes in the myocardium at that time. In individual cases the presence of C5b-9 in sufficient concentrations would depend on the rate of diffusion of complement substrate into the infarcted area, i.e. the rate of reperfusion. The sensitivity and specificity of the method to detect the early myocardial necrosis is the other factor that could be improved.

This study shows that there were relevant differences between the 2 brands of ab and the 3 different visualization techniques. The combination of polyclonal ab and the AP method resulted in satisfactory detection of C5b-9 in tissue sections. The reasons for the differences between

different brands and methods are not well understood, except for the higher sensitivity (and lower specificity) of polyclonal ab in general which depends on the affinity for multiple epitopes on the antigen. The ABC method with enhancement of the DAB reaction product with Co-Ni gave equally satisfactory results, and could also be used with the monoclonal ab. However, the method is more costly and elaborate, and in some cases a higher unspecific background staining was seen.

The immunohistochemical method (polyclonal ab and AP method), has an acceptable sensitivity for CN, i.e. 97%. The specificity of the method defined as the percentage of C5b-9 positive lesions that corresponded to CN in parallel sections was 44%. This could be interpreted either as if 56% of the sections contained artefacts or deposits of C5b-9 for other reasons than ischemic necrosis or it could mean that these positive lesions represented early ischemic areas not yet visible in the hematoxylin-eosin stain. The latter view is supported by the fact that in many such lesions, subtle changes were seen in the hematoxylin-eosin stain, i.e. eosinophilia, edema and variable nuclear hyperchromasia. These changes were not systematically evaluated due to the subjective nature of interpretation. However, we cannot estimate the exact specificity of the method without controlled experimental studies. It has been discussed whether the CB lesion is a true marker of myocardial ischemia, and if it is a reversible or irreversible injury (Karch and Billingham 1986). The fact that these lesions were variably positive for C5b-9 ab does not solve that dispute. Some of the CB seem to occur agonally, for example after resuscitation efforts (Karch and Billingham 1984), which does not exclude irreversible damage, but without a vital reaction in the form of complement activation. By excluding the CB that were not C5b-9 positive, it appears possible to sort out the agonal CB. CB occurring at the edges of infarctions (CN) were invariably positive with C5b-9 ab, making it plausible that these CB are older. The problem arises when the CB are seen isolated. Here the immunohistochemical detection of C5b-9 can help. By application of the method described here in cases of sudden death, with or without CAD (Figs. 2 and 3) it seems possible to detect recent infarctions in more cases in both groups, +23% in the CAD group and +11% in the non-CAD fatalities (Fig. 3). In the CAD group an even higher percentage of C5b-9 positive lesions without corresponding changes in the routine stains were seen (+34%). This result should be interpreted with caution because the specificity of the method is not known, but it is tempting to regard these lesions as very early infarctions, but how early we cannot know. In a recent work Thomsen and Held (1995) claimed that C5b-9 could be seen as early as 40 min. If this is a correct interpretation of our results it would be an important progress in the diagnosis of early myocardial infarction in sudden cardiac death. In cases of cardiac defibrillation it would also be possible to more easily discriminate between intravital and agonal CB lesions.

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