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ORIGINAL ARTICLE

Reaction patterns of pulmonary macrophages in protracted asphyxiation

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Abstract Do long periods of asphyxiation trigger the proliferation of pulmonary macrophages and the formation of giant cells? Three groups have been defined: six autopsy cases with time periods of suffocation >25 min (long protracted asphyxiation), eight cases with estimated time periods of suffocation 10–25 min (short protracted asphyxiation) and nine cases where death had occurred immediately (very severe trauma). The stain used was haema toxylin and eosin (H&E), and the immunohistochemical stainings were performed using antibodies CD 68, MRP 8, MRP 14 and NP 57. The intraalveolar macrophages and giant cells were counted in H&E sections. For the immunohistochemical stainings, a scoring was used in order to compare the groups. In protracted asphyxiation, the number of intraalveolar macrophages was definitely elevated. A significant increase of giant cells was observed in the cases of long protracted asphyxiation. CD 68 showed clearly elevated numbers in both asphyxiation groups. Early-stage macrophages are significantly increased in protracted asphyxiation. With increasing time periods of asphyxiation, the results become more significant. The results show that the length of the agony period stimulates the proliferation of pulmonary macrophages and the formation of giant cells.

Keywords Protracted asphyxiation · Lungs · Macrophages · Giant cells · Early-stage macrophages · Granulocytes

Introduction

Suffocation can be a difficult diagnosis because the pathological anatomy of hypoxia induced damages (hypoxidoses) can have several reasons, e.g. internal disease or their complications, metabolic dysregulations, intoxications and external reasons. If the differential diagnosis of "internal" was excluded, then, there still remains a large variety from "mechanical" to "atmospheric". It is therefore extremely desirable to prove the cause of the suspected suffocation type, such as bleeding in the neck muscles in suspected manual strangulations.

Unfortunately, concomitant injuries can be missing in cases of soft tissue covering, asphyxiation caused by rebreathing or non-traumatic thorax compression. In these groups, forensic medicine is eminently dependent on additional evidence.

For some time, past experts have become attentive to the reaction of alveolar macrophages. Janssen [14] as well as Janssen and Bärtschi [15] verified the increase of macrophages and giant cells in human pathology and in animal experiments. Hereafter, there have occurred several studies with opposing results: some found an activation of macrophages especially in cases of protracted asphyxiation [7, 23]; others confuted these reactions [2, 3, 9].

In recent years, several markers have been described allowing for a classification of macrophages into subgroups. With these and additional tools, we have attempted to perform a reevaluation of this area by examining quantitatively two well-defined groups of protracted asphyxiation in relation to a control group.

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Material and methods

Three groups have been defined:

1. Deaths due to long protracted asphyxiation, estimated agony period above 25 min, $n=6$ (Table 1, "Cases with protracted asphyxiation, long agony period")
2. Deaths due to short protracted asphyxiation, estimated agony time 10–25 min, $n=8$ (Table 1, "Cases with protracted asphyxiation, short agony period")
3. Immediate deaths as controls, agony period< 1 min, $n=9$ (Table 2, "Controls with very severe trauma")

Table 1 Individual data for the groups with protracted asphyxiation, long agony period and short agony period

Case	Age, years	Sex	Cause of death	Period of agony, min	Period of autolysis, h	Petechiae[1]	Cyanosis	Emphysema	Signs of asphyxia death
Cases with protracted asphyxiation, long agony period									
1	24	M	Rebreathing (hood) loosely corded	25	24	0	++	++	Hyperemia of liver and kidney
2	26	M	Rebreathing (garbage bag) loosely corded	30	48	++	0	++	Liquid blood
3	81	F	Rebreathing (plastic bag) loosely corded	30	24	+	+	++	Hyperemia of liver
4	77	M	Rebreathing (plastic bag) loosely corded	30	15	+	0	+	Liquid blood
5*	44	F	Rebreathing (garbage bag) loosely corded and smothering (pillow after 30 min)	35	12	0	0	++	Liquid blood
6	0.5	M	Smothering (plastic cover)	25	24	+++	+++	+	Liquid blood
Cases with protracted asphyxiation, short agony period									
1	58	M	Smothering (pillow) and burking	10	5	+	0	+	Liquid blood, hyperemia
2	64	F	Smothering (pillow)	15	24	+++	+	++	Liquid blood, hyperemia
3	3.5	M	Thoracic compression	20	24	+++	++	++	Liquid blood, hyperemia (kidney and spleen)
4	45	M	Rebreathing (plastic bag) firmly corded	20	12	+	+++	++	Liquid blood
5	32	F	Rebreathing (plastic bag) firmly corded	20	12	0	+	+	Liquid blood
6	33	F	Smothering (towel)	15	6	+++	+	+	Liquid blood
7	24	F	Rebreathing (plastic bag) firmly corded	20	24	++	0	+	Liquid blood, hyperemia
8	35	M	Rebreathing (plastic bag+ dilution of nitro)	10	24	0	++	+++	Liquid blood, hyperemia (liver and kidney)

[1] 0=non-described, +=isolated appeared, ++=moderately appeared, +++=distinctively appeared

5*: Observed by an independent person (see text)

Table 2 Controls with very severe trauma

Case	Age, years	Sex	Cause of death	Aortic rupture	Period of agony	Period of autolysis, h
1	59	M	Run over (railway) with decapitation	+	Seconds	36
2	45	F	Run over (railway) with decapitation	−	Seconds	48
3	20	M	Run over (railway) with decapitation	−	Seconds	24
4	?	F	Run over (railway) with decapitation	−	Seconds	48
5	52	M	Plane crash with polytrauma	+	Seconds	24
6	20	M	Run over (railway) with decapitation	−	Seconds	72
7	65	F	Run over (railway) with decapitation	+	Seconds	7
8	22	M	Run over (railway) with decapitation	−	Seconds	48
9	1.2	M	Electrocution in bathtub	−	Seconds	13

Age distribution, average ages, gender distribution and smoking habits were roughly comparable in the groups (Tables 1 and 2).

All agony times were estimated according to the statements of the autopsy reports. In the first group, there existed one case (cf. Table 1, case 5) where the time scale was described by an observer. This time was considered as the limitation between both groups: in group 1, the heads of the victims were usually put in plastic bags of considerable sizes with only a loose or no ligature around the neck, thus allowing for exchange of the air inside the bag with the air surrounding. In group 2, the time of agony was shorter than in group one. Some of the victims were found with their head in a plastic bag with a very firm ligature around the neck, so there was no or much reduced chance of air exchange. Two were pressed on a pillow or some towels, and one died because of the compression of the thorax (Table 1).

In group 3, we concluded on immediate deaths because of very severe trauma, i.e. typically decapitation with or without an aortic rupture, with one exception (case 9, Table 2).

Histology, histochemistry

Five-micrometre sections were taken from paraffin blocks of all existing specimen per case, including peripheral (subpleural) and central areas (cf. Table 3). The routine staining was H&E. For histochemistry, we have applied the avidin–biotin-complex method using the following antibodies: CD 68, MRP 8, MRP 14 and NP 57 [20].

Properties and specificities of the used antibodies

CD 68 (PG-M1)—dilution after chess board titration 1:50, detects all macrophages and monocytes [8, 12].

MRP 8 and MRP 14, used in final dilutions of 1:200 and 1:400, respectively, detect calcium binding proteins MRP 8 and MRP 14 which are expressed in young monocytes, blood macrophages and granulocytes. Mature (tissue) macrophages do not express these proteins. These proteins are indicative of an early inflammation, acute processes expressing MRP 14, subacute ones MRP 14 and MRP 8 in combination [21, 24].

NP 57 (final dilution 1:50) detects neutrophils and their precursors [1]. This staining was used as a control against MRP 8 and MRP 14. It also gives a hint for an increased activation of the marrow.

Microscopic evaluation

The number of specimens taken per case varied between two and six (cf. Table 3). All of them were H&E stained and microscopically examined. A substructure of them was stained for immunohistochemistry (IH; CD 68, MRP 8, MRP 14, NP 57) and fully examined (cf. Table 3). Thereafter, we have performed cell counting and/or semiquantitative evaluation. All fields were photographed, and the counts/scoring were performed on the prints. The structure of the fields selected is shown in Tables 3 and 4.

The structure of the selection was as follows: in a preselection phase at either ×100 (IH) or ×200 magnification (HE), an appropriate area, central and peripheral, was chosen. Negative criteria of this selection were: bleeding, oedema, atelectasis and expressed emphysema; positive criteria were: slightly overinflated areas (see e.g. Fig. 2). This preselection had the advantage that erroneous cell counts due to bleeding, atelectasis and also to insufficient statistics (strong emphysema) were avoided.

Cell counts Four areas per case were examined, i.e. two central and two peripheral areas at ×200; each of them was further subdivided into three fields, each at ×400 (altogether 12 fields per case). Every ×400 field was cell-counted, and the obtained results were extrapolated to the whole area of the ×200 section. Cell counts were performed by two independent observers, who were blinded against the case group. Blinding against the origin (central/peripheral) was

Table 3 Structure of staining procedures and evaluation procedures

		Very severe trauma $n=9$	Short protracted asphyxiation $n=8$	Long protracted asphyxiation $n=6$
H&E	Specimens (sum of all cases)	∑=36	∑=32	∑=27
		22p/14c	21p/11c	15p/12c
	Evaluated fields (×400) per case	6p/6c	6p/6c	6p/6c
Immunhistochemistry (CD 68, MRP 8/14, NP 57)	Specimens (sum of all cases)	4×∑=20	4×∑=19	4×∑=16
		4×12 p/8c	4×11p/8 c	4×9p/7c
	Evaluated fields (×200) per case	4×1p/1c	4×1p/1c	4×1p/1c
Megakaryocytes (MRP 8)	Evaluated fields (×200) per case	Ø=20	Ø=20	Ø=20
		10p/10c	10p/10c	10p/10c

p peripheral, *c* central

not possible because of the different structures. An interobserver difference of >20% was noticed in 18.4% of the cases. These were re-discussed with an experienced histopathologist based on the original photographs. A second-blinded valuation always resulted in differences less than 8%. Afterwards, the mean values were taken. Only alveolar macrophages were evaluated. The definition "giant cell" required a minimum of two nuclei.

Scoring A framework for semiquantitative scoring was established. After cell counting of representative prints, a table was established with the correspondences between numbers and scores (cf. Table 4). This table was accompanied with exemplary photographs for all scores. In the phase of semiquantitative evaluation, a given photograph was compared with the cell-counted photographs and thus assigned to the appropriate grade. Each preselected area (×100) was subdivided into smaller fields (×200) which were then photographed and scored. This scoring approach was further checked for representativeness: in each two cases of a case group, six areas were preselected at ×100, i.e. central and peripheral, and further subdivided into three fields at ×200 and the mean values taken. The scores as obtained by the "extended screening" were the same as those from the original approach. An interobserver-variability was noticed in two cases. These were re-discussed and repeated with no differences remaining.

The cell findings were differentiated between intra-alveolar and interstitial. Among the interstitial cells, we did not discriminate between intracapillary and interstitial as this is microscopically sometimes very difficult, and there also exist transient forms, i.e. cells in the stage of emigration.

The intraalveolar cells were easy to diagnose because they were positive in CD 68 and H&E. A mix-up with desquamated epithelial cells was therefore impossible.

Megakaryocytes were counted at ×400 on sections stained with MRP 8. 20 vision fields per case were evaluated and the mean values and variations calculated in order to check for homogeneity of the distribution (cf. Table 5).

Results

CD 68 High scores (+++) were observed only in cases of asphyxiation (Fig. 1a). The cells were found intraalveolar and interstitial. Furthermore, the reaction strength was definitely stronger in the groups of protracted asphyxiation (cf. Figs. 1a, 2a, b, 3a, b).

MRP 14 High scores were observed only in groups of asphyxiation. The remaining scores were less discriminative. There also was a stronger reaction of the cells in cases of protracted asphyxiation (cf. Figs. 1b, 3c, d).

Table 4 Score of the immunohistochemical stainings

Grading	CD 68 (cells/field)	MRP 14 (cells/field)	MRP 8 (cells/field)	NP 57 (cells/field)
0	0–3	0–3	0–3	0–3
0 to +	5–15	3–10	3–10	3–10
+	15–30	10–18	10–18	10–20
++	30–55	18–25	18–25	20–30
+++	>55	>25	>25	>30

Table 5 Results of the megakaryocytes cell counting

Very severe trauma	Ø number of megakaryocytes/vision field	Slow asphyxiation (agony <25min)	Ø number of megakaryocytes/vision field	Very slow asphyxiation (agony >25min)	Ø number of megakaryocytes/vision field
Case 1	0.2	Case 1	1.95	Case 1	3.75
Case 2	0.65	Case 2	2.5	Case 2	3.1
Case 3	0.7	Case 3	3.45	Case 3	4.25
Case 4	0.6	Case 4	2.95	Case 4	1.55
Case 5	1.1	Case 5	2.6	Case 5	3
Case 6	0.75	Case 6	3.6	Case 6	4.55
Case 7	0.55	Case 7	3.2		
Case 8	0.8	Case 8	2.25		
	Ø=0.67		Ø=2.81		Ø=3.37

The average of 20 vision fields (×400)

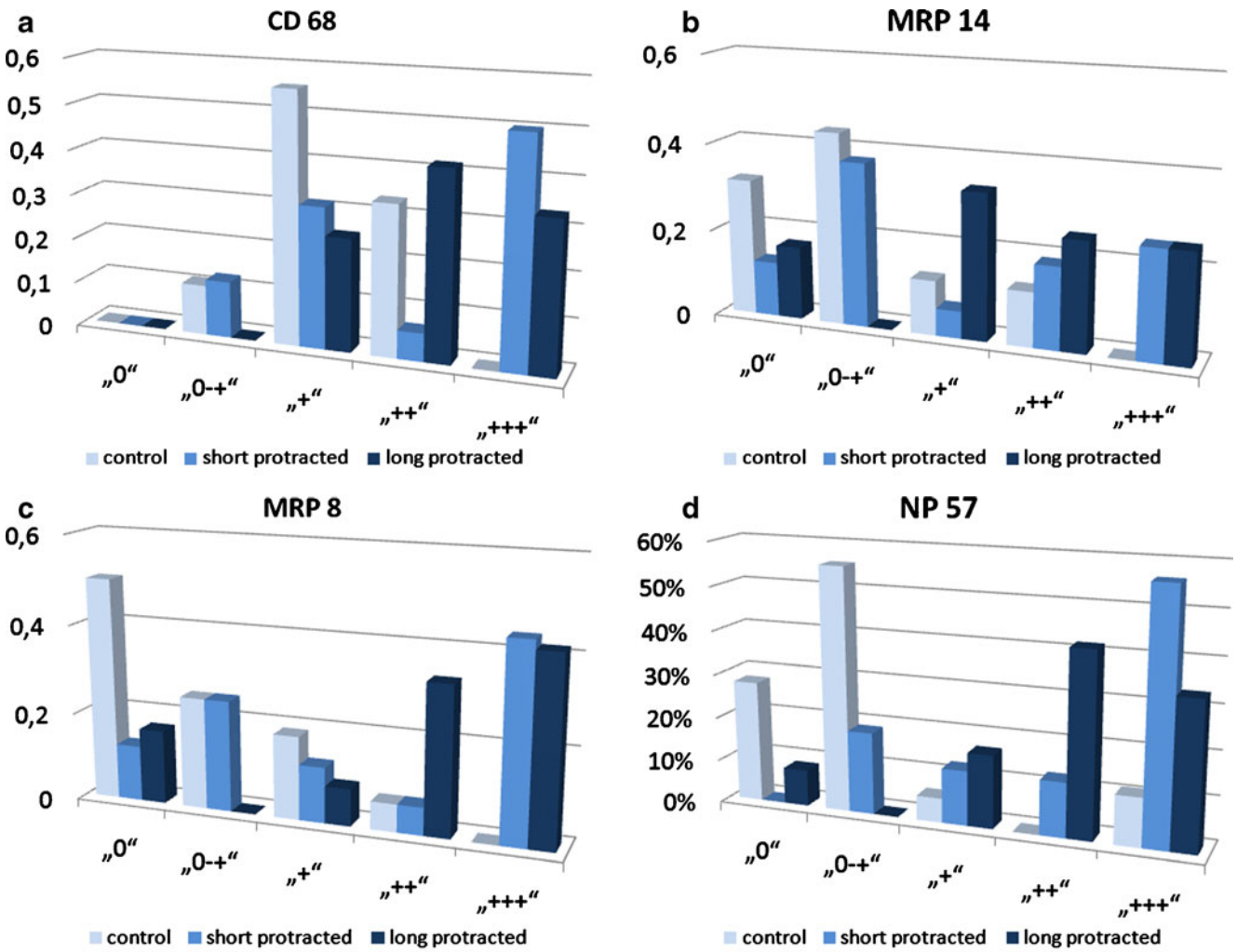

Fig. 1 Results of the IH scoring in percentages/group. **a** CD 68, **b** MRP 14, **c** MRP 8, **d** NP 57. The data are composed of the summation of all data evaluated, i.e. peripheral and central. The case/specimen/field statistics are as shown (Table 3)

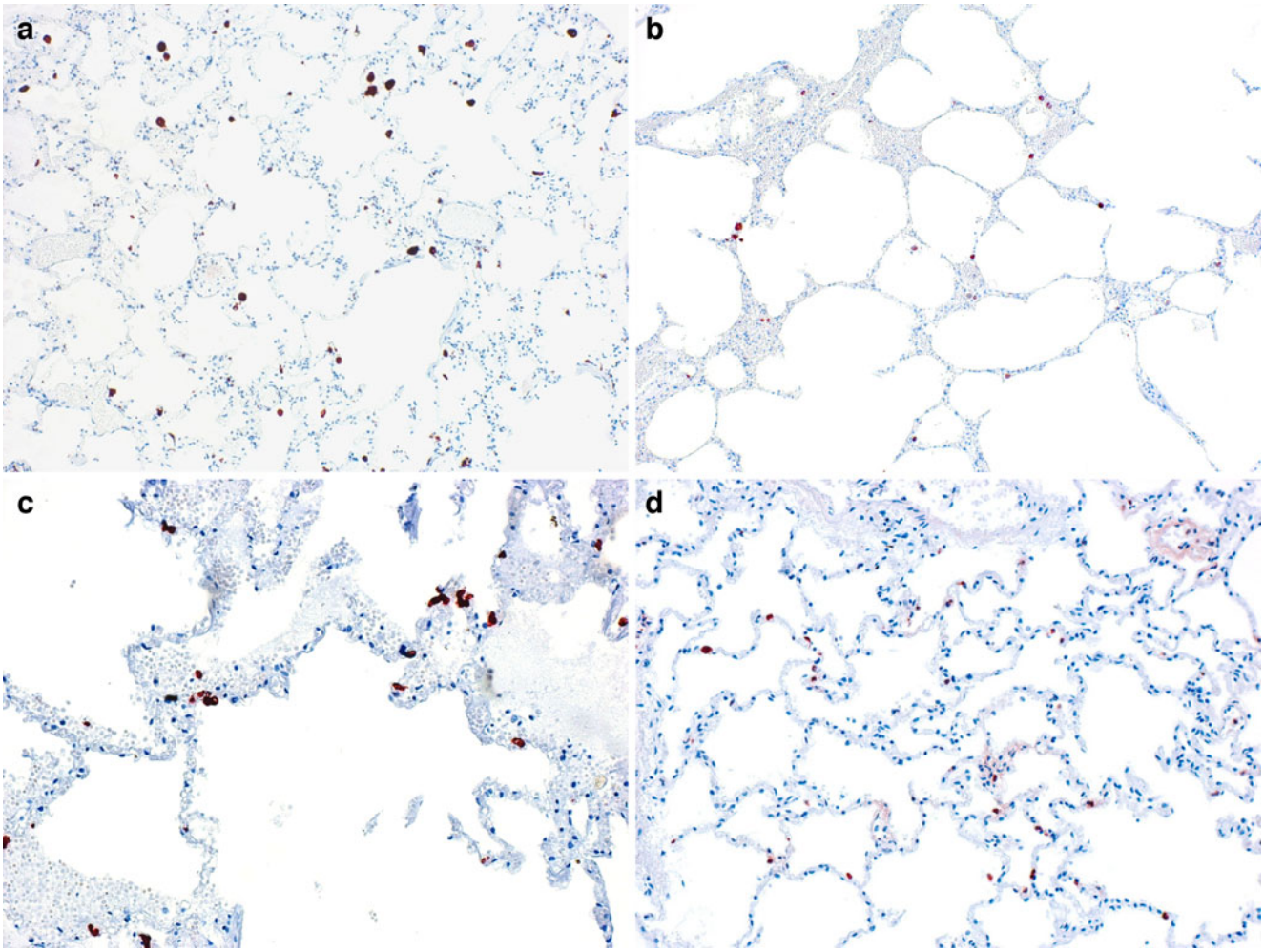

Fig. 2 Immunohistochemical stainings. **a** CD 68 1:100, protracted asphyxiation. **b** CD 68 1:100, very severe trauma. **c** MRP 8 1:200, protracted asphyxiation. **d** MRP 8 1:200, very severe trauma

MRP 8 Here as well, the highest score was discriminative between controls and cases (cf. Figs. 1c, 2b).

NP 57 The cases of asphyxiation predominantly reached scores of ++ and +++. Thus, this marker was discriminative between controls and cases. But the highest score was also reached by one control case (cf. Fig. 1d).

Megakaryocytes Megakaryocytes showed a significant increase in cases of asphyxiation (Table 5). An average of 0.67 as found in the controls is contrasting with an average of 2.81 found in cases of slow protracted asphyxiation and 3.37 as found in cases of long protracted asphyxiation (cf. Table 5).

Specificity, topology Whilst CD 68 cells were found in both compartments, alveolar and interstitial, all other cells were exclusively or nearly exclusively found in the interstitium. A differentiation between intracapillary and interstitial was often difficult to make. Therefore, we have applied a summary of these two locations with the term interstitial. However, the shape of the cells is often sausage-like, and sometimes, they are showing the typical shape when leaving the capillaries, i.e. with a waste. These findings do indicate that a significant proportion of these cells are still in the capillaries. In all histochemical stainings, no difference existed between central and peripheral fields. We have also checked whether there existed cross reactions between the antibody-mediated reactions: CD 68-positive cells showed no reaction with one of the other antibodies. Between two consecutive sections, we have evaluated the antibodies MRP 14 and MRP 8 with only a small overlap between them. The same holds for true with a comparison of MRP 8/NP 57. There existed sections with relatively many MRP 8 cells and the subsequent ones with no or nearly no NP 57 cells.

The inter-field variation of the H&E cell counts was low within the peripheral fields and within the central fields of the same case, respectively, higher numbers of macrophages and giant cells in the cases than in the controls. These differences were typical cell counts of the three fields

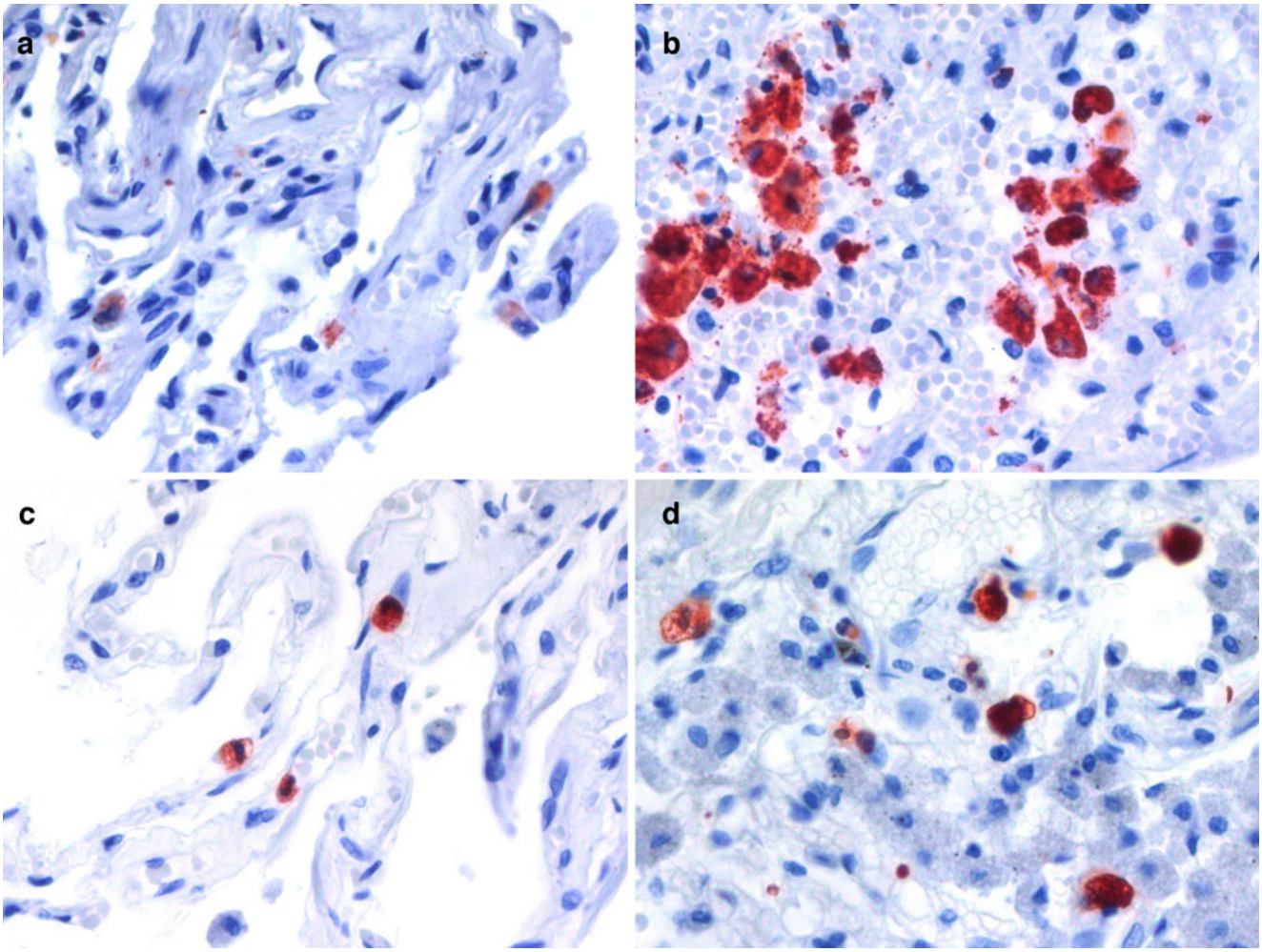

Fig. 3 Differences of the strength of the staining between controls and cases. **a** Very severe trauma. CD 68, 1:400 **b** protracted asphyxiation. CD 68, 1:400 **c** very severe trauma. MRP 14, 1:400. **d** Protracted asphyxiation MRP 14 1:400

(×400) in a given case were e.g. I: 21.5; II: 22; III: 21.5 or I: 8; II: 5; II: 7. In conclusion, all cell counts were associated with significantly more expressed in the peripheral fields. Both asphyxiation groups were associated with elevated macrophage counts whereas the giant cells were more frequent in long protracted asphyxiation (cf. Figs. 4, 5 and 6). There existed no age or gender relation, i.e. young persons had no elevated cell counts (cf. Fig. 7).

Discussion

Protracted asphyxiation is associated with complex changes and thus with a shift of pulmonary macrophages:

1. Firstly there occurs a significant increase of intraalveolar mature macrophages, CD 68-positive. This increase is expressed more in the periphery and can only be derived from the "interstitial macrophage pool". In relation to the controls, the cell counts become roughly doubled.
2. There also occurs an increase of (intraalveolar) giant cells with a significant difference between "short protracted asphyxiation" and "long protracted asphyxiation". Their numbers became roughly doubled with each step, i.e.: controls<short protracted<long protracted. Here as well, these differences are more expressed in the periphery than centrally.
3. Looking after "young macrophages", strong reactions (++) and very strong reactions (+++) are either exclusively (+++) or predominantly associated with protracted asphyxiation.
4. Also, other cells (granulocytes) that are usually associated with shock or periods of extreme stress show a very similar reaction pattern with strong and strongest reactions only or predominantly protracted asphyxiation. Microembolism and its subtypes accompanying the granulocytes have not been observed [4].

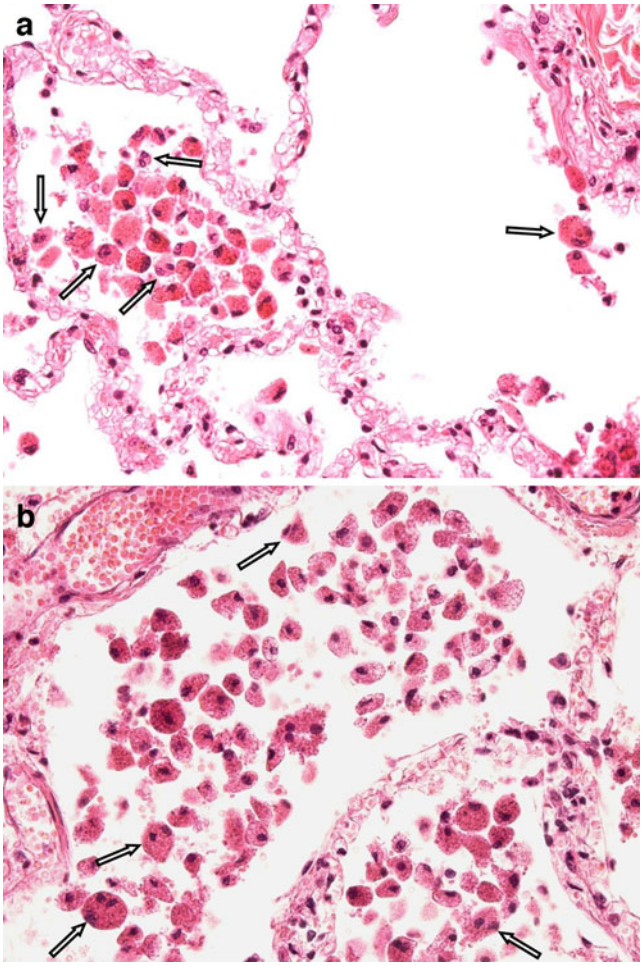

Fig. 4 HE. Intraalveolar macrophages and giant cells in casaes of protracted asphyxiation (giant cells are *arrow-marked*). 1:400

5. Megakaryocytes, indicative for either anaemia or hypoxia, showed the strongest increase under all cells tested, i.e. a fourfold to fivefold increase of the cell numbers in protracted asphyxiation.

In other words, one can say that the doubling or tripling of intraalveolar macrophages and giant cells are regularly associated with a significant increase of the number of

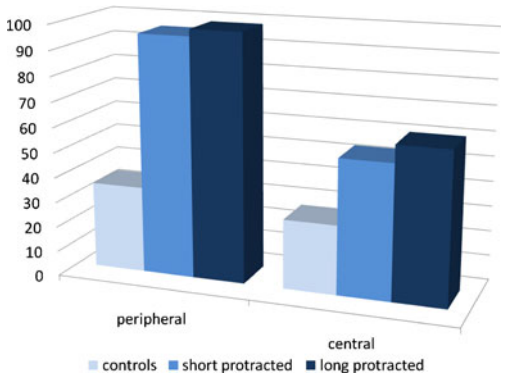

Fig. 5 HE. Mean values of counted macrophages in the areas (×200)

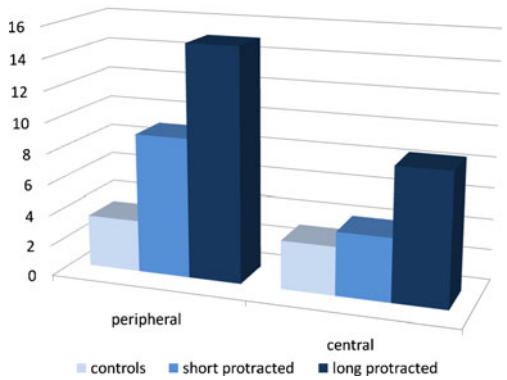

Fig. 6 HE. Mean values of counted giant cells in the areas (×200)

"young macrophages" and other cell types that are also hypoxia-associated.

Since the intersection-, the interfiled- and the interobserver-variability of the evaluated areas were low, and since the number of the sections and fields examined per case was relatively high, we consider our results obtained as representative for the entire lung.

Some authors have doubted the specificity of the increase of macrophages by stating this is not limited to asphyxiation only [2, 3, 9]. Their proliferation can be triggered by, e.g. inhalation of dust, cigarette smoke and infections [6]. Therefore, controls can also be positive. An increase of macrophages, however, cannot be an artefact of the post-mortem period, which is for the following: CD 68 is a marker of macrophages and does not stain pneumocytes. Alveolar macrophages migrate on the alveolar surfaces and eat all types of foreign material, detritus, etc. They are there. On the contrary, pneumocytes are epithelial cells (P I) or cells producing and secreting the surfactant (P II). They can be desquamated in the post-mortem period but still are distinguishable from macrophages. The considerably higher number of macrophages in cases of

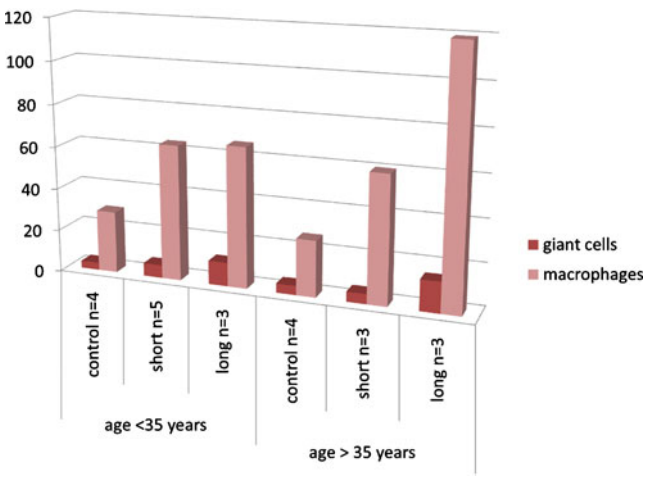

Fig. 7 Comparison of the age cohorts

asphyxiation, detected by staining with CD 68, however, indicates the existence of mechanisms in asphyxiation that can stimulate macrophages: the MCSF (macrophage colony stimulation factor) can be stimulated under conditions of hypoxia [13]. The same is true for an increase of catecholamines and phospholipids [11]. It has also been shown that asphyxia-related increase of histamine is accompanied with the formation of pores and vacuoles in the pulmonary circulation [16]. Also, an increased expression of the urokinase plasminogen receptor as induced by hypoxia [18] can induce an increased mobilisation of macrophages into the intraalveolar spaces [10].

There obviously exist quantitative differences in the mobilisation between central and peripheral; reasons could be associated with other differences between central and peripheral: the distance and the number of branching of the bronchial tree are much smaller for central alveoli than for peripheral ones, i.e. under extreme conditions of hypoxia, the central ones are relatively better oxygenised than the peripheral zones. According to the law of Euler–Liljestrand, the perfusion is controlled by autoregulation in small units (the acini) and follows the oxygen supply. The impact of this mechanism under hypoxic conditions is not yet fully understood. The acute pulmonary emphysema which accompanies the asphyxiation is less expressed in the central regions. Another possible reason is the fact that the so-called interstitial macrophage pool can vary in its capacity.

All these aforementioned facts can have an influence on the differences between central and peripheral zones.

The increase of early-stage macrophages seems to be asphyxiation-induced. The markers MRP 8 and MRP 14 are expressed during the maturation and transition of blood monocytes into tissue macrophages [24]. During inflammatory processes, they are expressed only after hours/days [23, 24], whereas in asphyxiation, there appear to exist some additional triggers that can advance the activation of macrophages [7] and considerably shorten the expression process of MRP 8/14: GM-CSF, LPS and proteinase C [22]. In conclusion, the application of MRP 8/14 indicates an earlier maturation and activation of macrophages under hypoxic conditions. Furthermore, it is easier to distinguish between a chronic stimulation of the macrophage/giant cell-system induced by smoke inhalation and an acute stimulation of macrophages/giant cells in cases of hypoxia.

The positive finding of the increased numbers of granulocytes detected with the antibody NP 57 leads to the idea of an increased activation of the bone marrow under the influence of hypoxia [7] which is in consistence with earlier investigators who have described immature bone marrow cells embolised in the pulmonary microcirculation in death due to strangulation [4, 5]. Furthermore, the increase of megakaryocytes, independent from the granulocytes, provided the idea of a proliferation and activation of bone marrow cells under hypoxic state [17] and can be a useful criterion to assure the diagnosis asphyxiation.

Protracted long asphyxiation (>25 min) is also associated with an increase of giant cells, while the number of giant cells in short asphyxiation is only slightly elevated. In other words, 20 min or less is obviously too short to create giant cells. This phenomenon has been described likewise by Janssen in 1963 and was proven experimentally [14]. Other studies have contradicted this [2, 3, 9]. A possible reason can be the selection of the controls which were, e.g. sudden heart deaths [9] and severe cerebral–cranial trauma [2]. These "controls" can survive for many minutes, and agony is usually accompanied with pulmonary hypoventilation.

In our study, however, death was due to very rapid exsanguinations by either aortic rupture or decapitation or similar, so it is assumed that a circulatory breakdown occurs within seconds. Another reason for the different results can be found in the definition of giant cells. While Betz et al. and Grellner et al. needed a minimum of three nuclei [2, 3, 9] our study, in accordance with Janssen [14], required a minimum of just two nuclei. As the normal size of a giant cell is much bigger than the section thickness of 5 μm, we often did not see the giant cell completely. This implies that a giant cell with two nuclei can also be one with three or four and one with three nuclei would include the ones with four or more nuclei.

Originally, it had been supposed that the formation of giant cells was due to endomitosis as an adaptation reaction [14, 15]. It is now accepted knowledge that, under the influence of oxygen depletion, macrophages can secrete cytokines and growth factors with the enhanced expression of surface markers with changes of the metabolism and the cell morphology [18]. Enhanced activities of the transcription factors NF–$_K$B [18] and of MCSF [13] can stimulate giant-cell formation by fusion and accumulation of cells [19]. Since this is not a cellular proliferation, the usage of proliferation markers such as Ki-67 [2] must necessarily fail.

The occurrence of virtually increased numbers of macrophages and giant cells do not prove suffocation since they also occur in other causes of death and are independent of the cause of death. But, high numbers of such cells can be a useful criterion to assure the diagnosis asphyxiation, e.g. in the case of a victim with morphological findings such as emphysema, petechiae, heavy congestion, fluid blood, etc. which let us suspect a suffocation death but shows a lack of conclusive tools (e.g. in soft tissue covering). To distinguish the macrophage/giant cells proliferation due to asphyxiation from other triggers (e.g. smoke inhalation), we strongly recommend looking after "young macrophages" and megakaryocytes and myelocytes to make this more specific.

Furthermore, these findings can help us to estimate the length of the agony as strong, and quantitatively positive reactions are associated with periods of agony >25 min.

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