Journal of Neuropathology and Experimental Neurology
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Vol. 52, No. 2
March, 1993
pp. 143–152

The Use of Antibodies Targeted Against the Neurofilament Subunits for the Detection of Diffuse Axonal Injury in Humans

M. Sean Grady, M.D., Mark R. McLaughlin, M.D., Carole W. Christman, Ph.D., Alex B. Valadka, M.D., Corinne L. Fligner, M.D., and John T. Povlishock, Ph.D.

Abstract. Axonal injury is a common feature of human traumatic brain injury. Typically, damaged axons cannot be recognized unless a patient survives the injury by at least 10–12 hours (h). Limitations associated with the use of the traditional silver methods have been linked with this inability to recognize early posttraumatic reactive axonal change. Recently, we reported that antibodies targeting the neurofilament subunits proved useful in recognizing early traumatically induced axonal change in traumatically brain-injured animals. Accordingly, in the present communication, we employed antibodies to detect at the light microscopic level the 68 kD Nf-L and 170–200 kD Nf-H neurofilament subunits in head-injured patients who survived the traumatic event for periods ranging from 6 h to 59 days. Antibodies targeting all of the above-described subunits revealed a progression of reactive axonal change. Antibodies to the 68 kD subunit proved most useful, as they were not complicated by concomitant immunoreactivity in surrounding nuclei and/or dendritic and somatic elements. These immunocytochemical strategies revealed, at 6 h postinjury, focally swollen axons which appeared intact. By 12 h, this focal swelling had progressed to disconnection, with the immunoreactive swelling undergoing further expansion over 1 week postinjury. These findings demonstrate the utility of the previously described immunocytochemical strategies for detecting reactive axonal change in brain-injured humans, particularly in the early posttraumatic course. More importantly, these methods also demonstrate in humans that reactive axonal change is not necessarily caused by traumatically induced tearing.

Key Words: Axonal swelling; Brain trauma; Human; Immunocytochemistry; Monoclonal antibodies; Neurofilament subunits.

INTRODUCTION

At present it is well recognized that diffuse axonal injury is a consistent feature of human traumatic brain injury, particularly those injuries involving rapid acceleration/deceleration of the brain, such as occurs with motor vehicle accidents, falls from considerable heights, or assaults (1–16). In recent communications we have noted that in various experimental animal models of traumatic brain injury, antibodies targeted to the neurofilament subunits not only proved helpful in the recognition of traumatically induced reactive axonal change but also provided insight into the possible pathogenesis of these reactive events (17–19). In this context, we noted that antibodies targeting the 68 kD subunit proved most useful. From the anatomical perspective, the use of antibodies targeted to this subunit was not complicated by excessive nuclear, somatic, or dendritic reactivity, which could mask the easy detection of reactive axonal change (17). Also, and perhaps most importantly, because an increase in the 68 kD subunit immunoreactivity was observed early in the sequence of reactive change, this observation provided insight into the possible pathogenesis of the reactive event. Specifically, it appeared that an unmasking and/or mobilization of the 68 kD subunit was pivotal in the genesis of continued reactive axonal change culminating in disconnection. After these observations were made in well controlled animal models of traumatic brain injury (17), it appeared reasonable that these issues should be critically evaluated in brain-injured humans coming to postmortem study.

In the present communication, we have employed antibodies to three neurofilament subunits, together with different harvesting and tissue processing procedures, to determine their usefulness in detecting axonal change in traumatically brain-injured humans. Additionally, through such approaches, we have attempted to gain a more complete assessment of the frequency with which these changes occur in human traumatic injury, as well as their localization in various regions of the brain.

MATERIALS AND METHODS

Postmortem analyses were conducted on ten severely traumatically brain-injured humans as well as one control patient. This control subject, a 33 year old male, was found dead, the possible victim of drug overdose with no evidence of traumatic injury. All traumatically brain-injured patients studied had an admission Glasgow Coma Score (GCS) of 7 or less, and had sustained either a motor vehicle accident involving acceleration/deceleration, an auto–pedestrian injury, or a fall. All brain-injured patients were admitted to a Level 1 trauma center either

From the Departments of Neurosurgery (MSG) and Pathology (CLF), University of Washington, King County Medical Examiner's Office (CLF) Seattle, Washington, and the Department of Anatomy (MRM, CWC, JTP) and Division of Neurological Surgery (MRM, ABV, JTP), Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia.

Supported by NIH Grant NS01371 (MSG) and NIH Grant NS20193 (JTP).

Correspondence to: John T. Povlishock, Ph.D., Department of Anatomy, Medical College of Virginia, Virginia Commonwealth University, P.O. Box 709 MCV Station, Richmond, VA 23298-0709

The material presented in this paper was presented in part at the 22nd Annual Meeting of the Society for Neuroscience, October 1992.

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TABLE 1
Traumatically Brain-Injured Patients

Interval between injury and death	Interval between death and fixation	GCS upon admission	Age and sex	Institution	Mechanism	CT findings	Management
6 h	7 h	3T	24 F	HMC	High speed MVA	Multiple petechial hemorrhages, subarachnoid blood	No surgery; ICP monitor
12 h	12 h	3	76 M	HMC	High speed MVA	Diffuse subarachnoid hemorrhage, several small punctate intraparenchymal hemorrhages (1st CT scan)	No surgery
30 h	40 h	3T	20 M	HMC	Auto–Pedestrian	Diffuse small to 2 cm parenchymal hemorrhages	No surgery
48 h	31 h	3	28 M	HMC	Fall from scaffold	Bifrontal contusion, left subdural and right intracerebellar hematoma	Surgery
60 h	12 h	5	47 M	HMC	High speed MVA	Putamenal hematoma	Multitrauma laparotomy
80 h	12 h	3T	18 M	HMC	High speed MVA	Multiple petechial hemorrhages	No surgery; ICP monitor
88 h	22 h	4	64 M	HMC	Fall from bar stool	Large subdural hematoma	Surgery
128 h	14 h	4T	41 F	HMC	Fall from horse	Multiple petechial hemorrhages, 2 × 3 cm clot in frontal lobes	No surgery
167 h	19 h	3T	41 F	HMC	Struck by bus while riding in car	Diffuse swelling, 2 cm; basal ganglia hemorrhage, multiple small contusions	No surgery
59 days	18 h	7	29 M	MCV/VCU	MVA	Right subdural hematoma	Late surgery

GCS = Glasgow Coma Score; T = Intubated; HMC = Harborview Medical Center; MCV/VCU = Medical College of Virginia/Virginia Commonwealth University; MVA = Motor Vehicle Accident; ICP = Intracranial Pressure; h = hours.

at Harborview Medical Center (HMC) (one of the University of Washington Hospitals) or at the Medical College of Virginia Hospital/Virginia Commonwealth University (MCVH/VCU). These patients were managed in an aggressive fashion consistent with established protocols to maintain physiological homeostasis and to control intracranial pressure. The traumatically brain-injured patients included in the study are identified in Table 1 by the duration of posttraumatic survival, interval preceding tissue fixation, GCS score, age, sex, institution, nature of their injury, CT findings, and management. Since all brain-injured patients were trauma victims, forensic postmortem examination was performed. Complete autopsy was performed, in most cases, within 24 hours (h) of death. These were performed either at the King County Medical Examiner's Office in Seattle, Washington, or at MCVH/VCU. The brains were removed, and tissue blocks were harvested from selected regions throughout the neuraxis. These regions included the corpus callosum, the frontal cortex and related subcortical white matter, basal forebrain, occipital lobe, basal ganglia, parahippocampal gyrus, midbrain, pons and medulla. Such tissue sampling was accomplished in traumatically brain-injured patients as well as the control case. Upon harvesting, all blocks were sectioned to a thickness of approximately 10 mm and immediately placed in a fixative composed of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer for 12 h. Those samples harvested at HMC were then transferred to 0.1 M phosphate buffer and immediately shipped with ice packs to MCVH/VCU. The majority of the tissues were sectioned on a vibratome at a thickness of 50 μm and collected in phosphate-buffered saline (PBS). In select cases, some tissue samples were cryoprotected in 30% sucrose, sectioned on a sliding microtome at a thickness of 30–50 μm, postfixed for 30 minutes (min) in 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, and placed in PBS. All sections harvested either from the vibratome or from the sliding microtome were immersed in 0.5% hydrogen peroxide in PBS to block endogenous peroxidase activity. Next, the sections were rinsed twice in PBS and processed for the immunocytochemical visualization of the 68 kD (Nf-L) or 170–200 kD range (Nf-H) neurofilament subunits.

All antibodies used were commercially produced. The NR4 antibody, which targets the 68 kD neurofilament subunit, was obtained from the Sigma Chemical Company, St. Louis, MO. The NR4 antibody has been characterized in detail (20–22). Antibodies SMI 31, SMI 32, and SMI 33 targeting selected epitopes primarily on the 170–200 kD neurofilament subunits were obtained from Sternberger Monoclonals Inc., Baltimore, MD. These antibodies have been fully characterized in published reports (23–26). The SMI 31 antibody reacts with extensively phosphorylated Nf-H, and to a lesser extent with Nf-M, while the SMI 32 antibody reacts with non-phosphorylated epitopes in Nf-H. Lastly, the SMI 33 antibody reacts with a non-phosphorylated epitope in both phosphorylated and non-phos-

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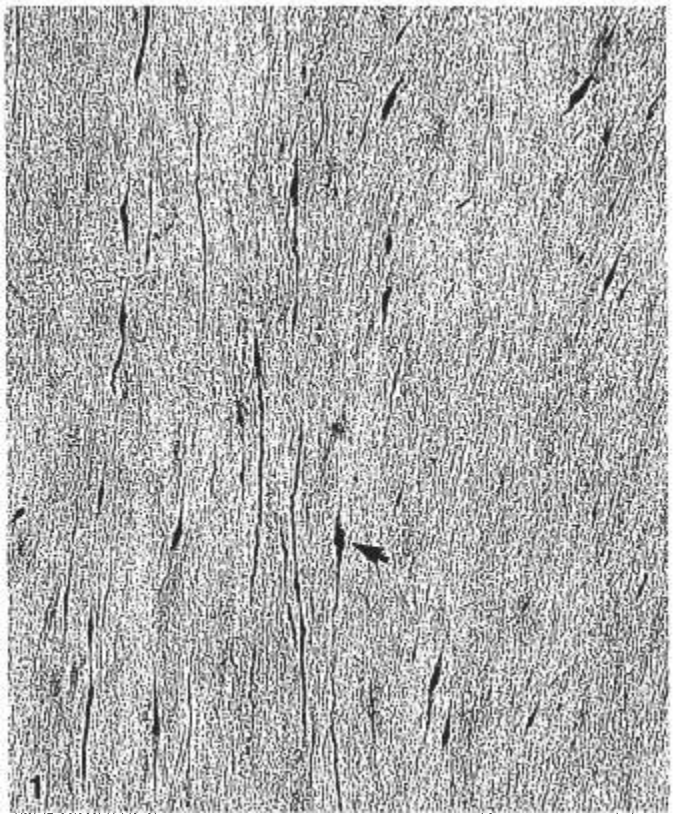

Fig. 1. At 6 h postinjury the NR4 antibody identifies reactive axons, one with a focal swelling (arrow), in the corpus callosum. Note that normal 68 kD immunoreactive fibers, which exhibit no focal swellings, can also be seen in the field. ×200.

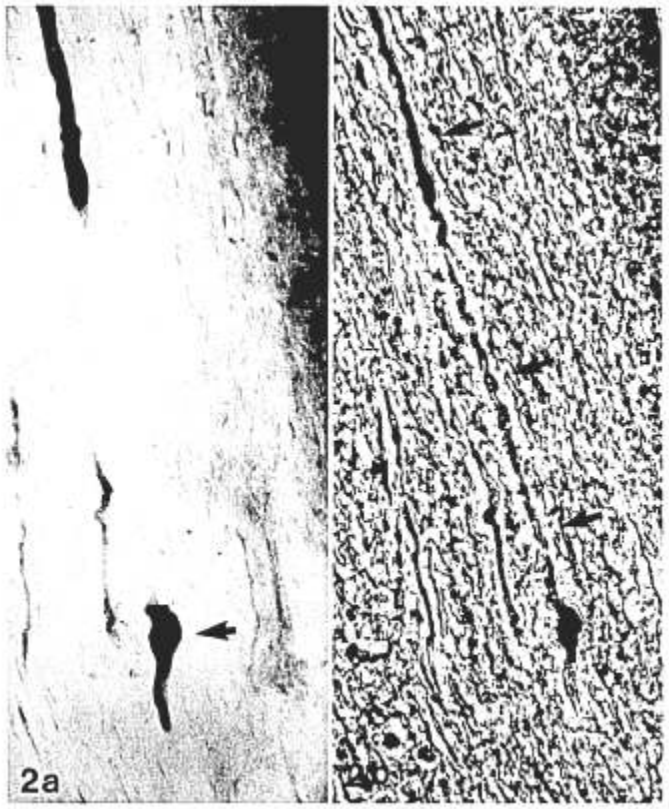

Fig. 2. In this light micrograph taken at slightly greater magnification than Figure 1, we can again recognize what appears to be a reactive axon (arrows) (a). Based upon the exclusion criteria noted in the Results section, this brightfield finding would not have been considered adequate evidence for reactive change. Yet, the use of phase microscopy (b) demonstrates that the axonal segments are in continuity (arrows), thus fulfilling our selection criteria. ×400.

phorylated Nf-H and, to a lesser extent, in Nf-M. For the 68 kD Nf-L subunit, the NR4 antibody was used at a dilution of 1:1,000. SMI 31, SMI 32 and SMI 33 were used at a dilution of 1:10,000. All sections were incubated with agitation for 16–18 h at 4°C in their respective primary antibodies diluted out accordingly in 1% normal horse serum (NHS) in PBS (TRIS for SMI 31). Following incubation in the primary antibody, the sections were rinsed 3 × 10 min in 1% NHS in PBS (1% NHS in TRIS for SMI 31) and then re-incubated for 1 h in biotinylated anti-mouse IgG (Vector, Burlingame, CA) in 1% NHS in PBS (1% NHS in TRIS for SMI 31). Next, the sections were rinsed 3 × 10 min in PBS (TRIS for SMI 31) followed by a 1 h incubation period in an avidin-biotin complex (ABC, Vector) made in PBS (TRIS for SMI 31). Both elite and standard ABC kits were used. For the visualization of the reaction product, the sections were reacted in 0.05% diaminobenzidine (DAB) and 0.01% hydrogen peroxide in 0.1 M phosphate buffer (TRIS for SMI 31) for 10–20 min.

Following the completion of the immunocytochemical procedures, the tissue was then processed in one of two fashions. Half of the tissue was mounted on glass slides, cleared, and coverslipped for light microscopic analysis, whereas the remaining tissue was osmicated and prepared for routine ultrastructural analyses. Following preparation, all cleared sections were examined by two investigators who were blinded to the case under evaluation. The investigators screened the sections and identified foci revealing reactive axonal change. In performing these analyses, rigorous exclusion criteria were used both in the traumatically brain-injured patients and in the control. Only those axons showing marked immunoreactivity were analyzed. Additionally, only axons whose course could be followed for considerable length were included in the data analysis. Thus, any presumed axonal abnormalities moving in a plane oblique or transverse to the field of investigation were not included in the data analysis.

RESULTS

Traumatically Brain-Injured Patients

General Immunocytochemical Observations: As in our initial observations in experimental animals, all antibodies targeting the neurofilament subunits detected reactive axons. However, as noted in our previous communication, antibodies targeting the 68 kD subunit appeared most anatomically useful. Not only were they capable of detecting reactive axonal change, but also their use was not complicated by concomitant immunoreactivity within related cell nuclei, somata, and dendritic trees. Because

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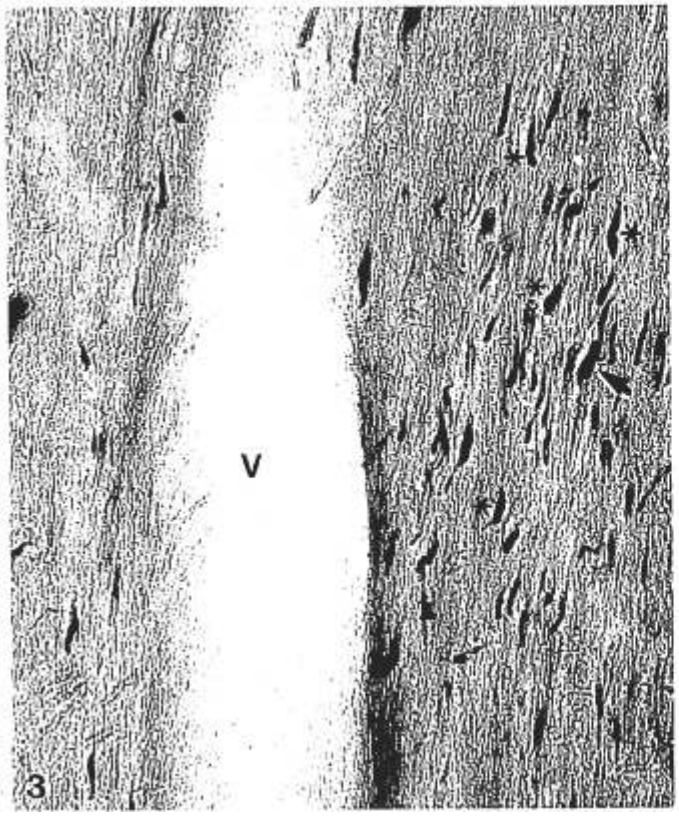

Fig. 3. At 12 h postinjury a 68 kD immunoreactive distended axonal segment (arrow) and swollen, disconnected axons (*) are seen paralleling a blood vessel (V) in the pons. ×200.

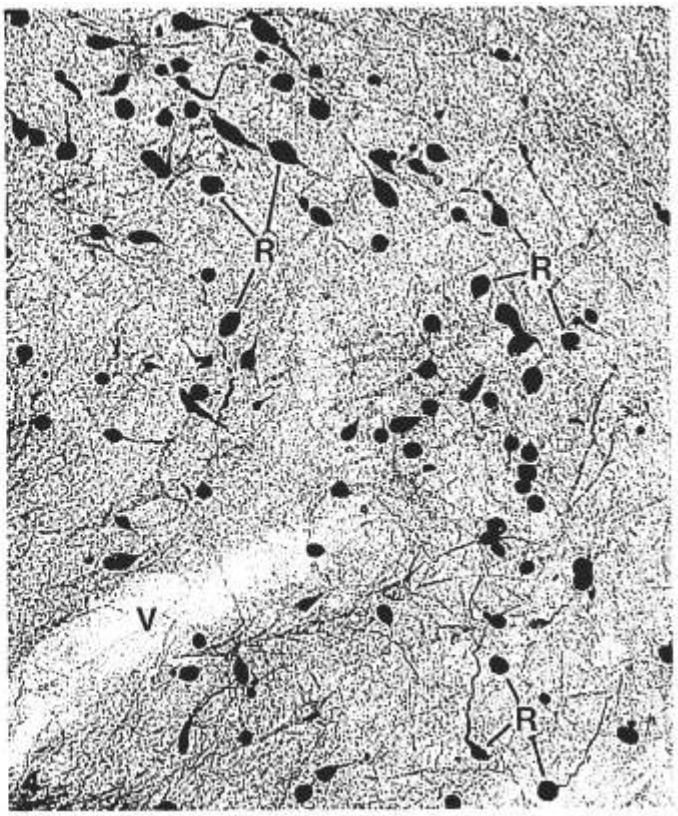

Fig. 4. At 88 h postinjury the NR4 antibody identifies numerous intensely dense immunoreactive swellings (R), some in relation to a blood vessel (V), within the hippocampus. ×200.

of the above properties, the 68 kD antibody was useful in detecting reactive axons not only late in the posttraumatic course when the change was most pronounced, but also in earlier time periods when reactive change was more subtle. In the early posttraumatic course (6 h postinjury), the antibodies to the 68 kD subunit typically detected within individual axons discrete focal swellings which occurred without disconnection (Figs. 1, 2). At such foci of axonal swelling the neurofilament immunoreactivity appeared increased, and, importantly, this immunoreactivity followed the contour change occurring in the focally distended segment. By 12 h discrete swellings were again identified; however, by now disconnection of the reactive axons apparently had occurred (Fig. 3). With continued survival (30 h–1 week) grossly swollen reactive axonal segments, which were now commonly disconnected, were easily identified (Fig. 4). With extended survival (59 days) comparable distended and disconnected reactive swellings were again seen.

Although reactive change was most easily identified using antibodies to the 68 kD subunit, the same reactive events were also visualized with the use of antibodies targeting the other neurofilament subunits. As noted above, however, the use of these antibodies was associated with considerable concomitant immunoreactivity visualized within related nuclei, somata, and dendritic domains. Such concomitant immunoreactivity frequently obscured the ready detection of the reactive axons, with SMI 31 and SMI 33 proving the most problematic, whereas SMI 32 provided information of intermediate quality (Figs. 5, 6, 7). These observations were reproducible, irrespective of whether the tissue had been sectioned on a vibratome or on a sliding microtome. Importantly, however, those tissues cryoprotected and sectioned on a sliding microtome offered the added advantage that they yielded more stable and consistent immunoreactivity throughout the entire section, most likely due to the enhanced fixation achieved through the use of the postfixation employed in conjunction with the cryoprotection strategy.

Specific Observations: Using the antibodies described above, we confirmed and extended many of our observations initially made in experimental animal models. Specifically, through the use of these antibodies, we observed that traumatically induced reactive axonal change in humans did not involve immediate tearing of the axon. Rather, at 6 h postinjury, focal intra-axonal accumulation of reactive neurofilament subunits appeared to set the stage for continuing reactive change (Fig. 8). By 12 h postinjury the continued expansion of the immunoreac-

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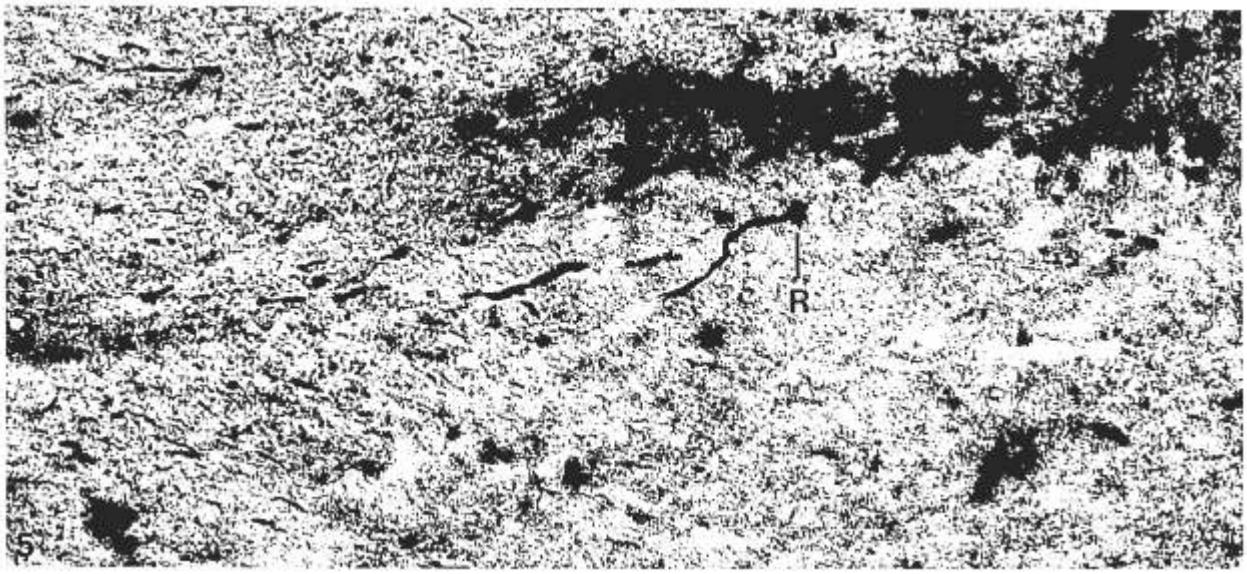

Fig. 5. With the use of the SMI 31 antibody, this light micrograph reveals an immunoreactive axonal swelling (R) whose ready identification is obscured by concomitant background immunoreactivity in the field (corpus callosum, 88 h postinjury). ×200.

tive neurofilament mass was correlated with evidence of disconnection (Fig. 9), suggesting that these reactive changes had now progressed to complete axotomy. Importantly, in this progression of reactive axonal change, some heterogeneity was observed, even within the same patient. Thus, at 12 h, reactive axons that were swollen and apparently disconnected could be seen adjacent to less dramatically swollen, intact axons. These intra-axonal events were identified in both small and large caliber axons. Also, importantly, these reactive axonal abnormalities were always visualized interspersed in fields displaying numerous apparently unaltered axonal profiles. At later stages in the posttraumatic course (>24 h), these reactive swellings exhibited further change in their configuration and appearance, with a non-immunoreactive cap of axoplasm now encompassing an expanded immunoreactive neurofilament core (Fig. 10). In some cases, lobulation of the axon was evident (Fig. 11).

These axonal changes frequently occurred at sites where the axons changed their anatomical course. Specifically, foci of axonal change were found just as axons either coursed over penetrating vessels (Figs. 3, 4, 11), turned

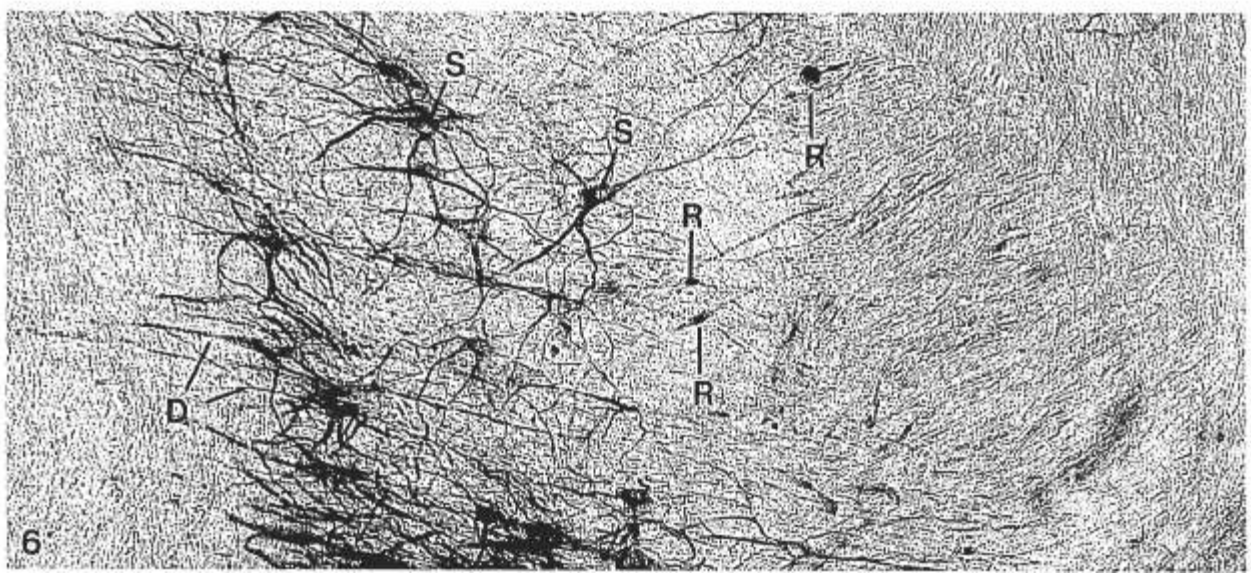

Fig. 6. With the use of SMI 32, some reactive axonal swellings (R) are visible. However, others are obscured by concomitant reactivity of somata (S) and dendrites (D) (pons, 88 h postinjury). ×200.

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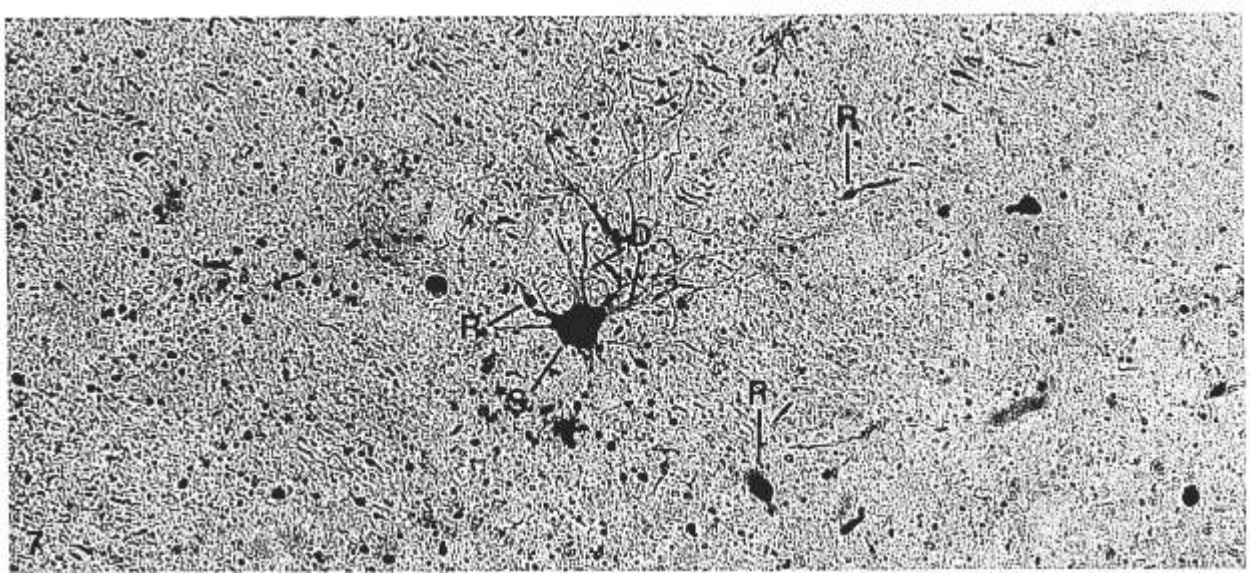

Fig. 7. SMI 33 reveals reactive dendrites (D) and a soma (S). Although reactive, swollen axonal segments (R) are visible, they are not as readily identified due to immunoreactivity demonstrated by the surrounding neuronal elements (basal ganglia, 80 h postinjury). ×200.

to enter target nuclei, or decussated within the brain parenchyma. Reactive axons were also seen where a change in tissue density occurred, such as at the gray/white interface.

While axonal injury was a consistent finding in all patients studied, differences in the overall frequency of reactive axonal change were evident in various brain regions sampled, a finding most likely attributable to differences in the nature of the initial traumatic insult. Although in all patients studied to date virtually all sampled loci showed evidence of reactive change, there was a general

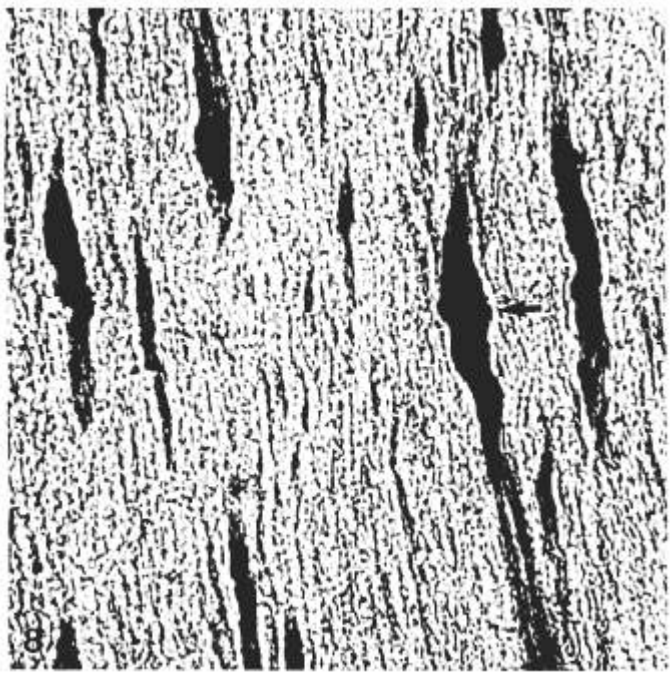

Fig. 8. This light micrograph shows with greater detail those early axonal changes seen with the use of the NR4 antibody targeting the 68 kD subunit. Note that a reactive axon with a focally distended area (arrow) is readily identified within the corpus callosum at 6 h survival. ×800.

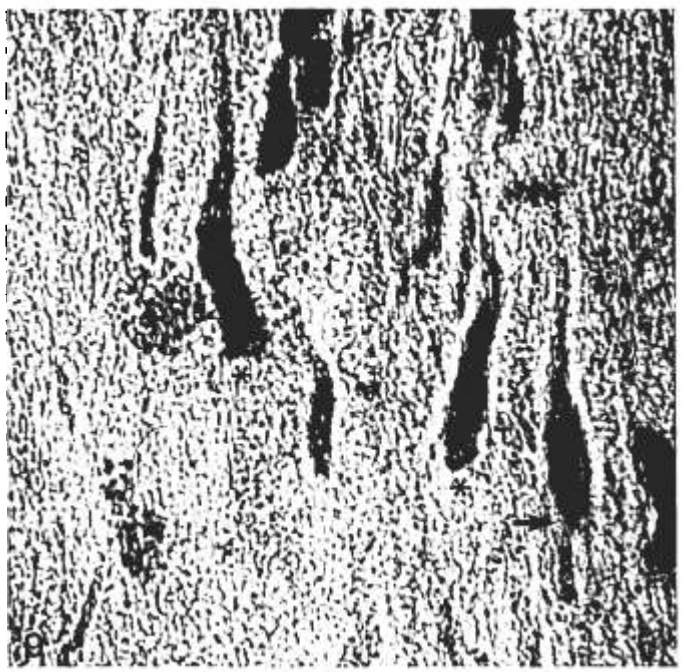

Fig. 9. With greater detail this light micrograph reveals, at 12 h survival, swollen 68 kD immunoreactive axons in the pons. Note that some appear to be disconnected (*), while another (arrow) appears to be intact. ×800.

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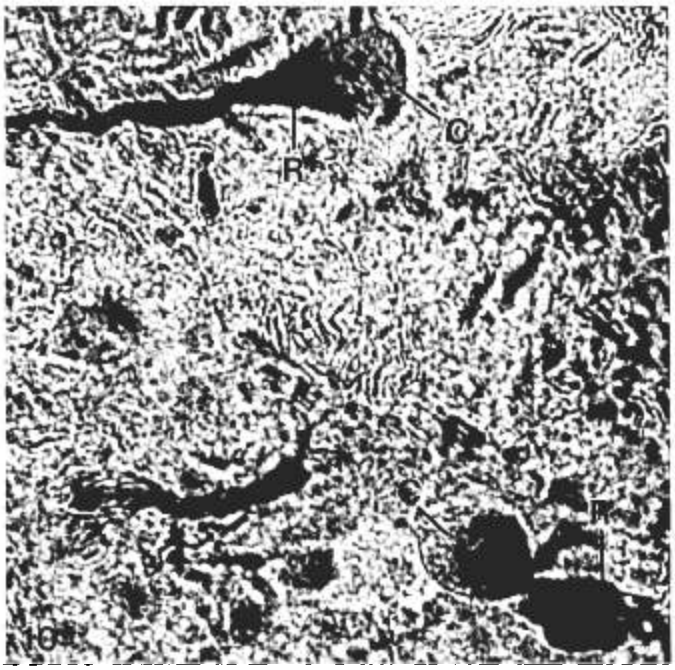

Fig. 10. At 80 h postinjury reactive axonal swellings (R) resembling those of classical description are seen in the basal ganglia through the use of the NR4 antibody. Also evident is the non-immunoreactive cap (C), which previous studies have shown to be devoid of neurofilaments yet laden with mitochondria and smooth endoplasmic reticulum. ×800.

Fig. 11. At 167 h survival disconnected axons exhibiting reactive swellings (R) and lobulation (L) are seen in relation to a blood vessel (V) with the use of the NR4 antibody (frontal subcortical white matter). ×800.

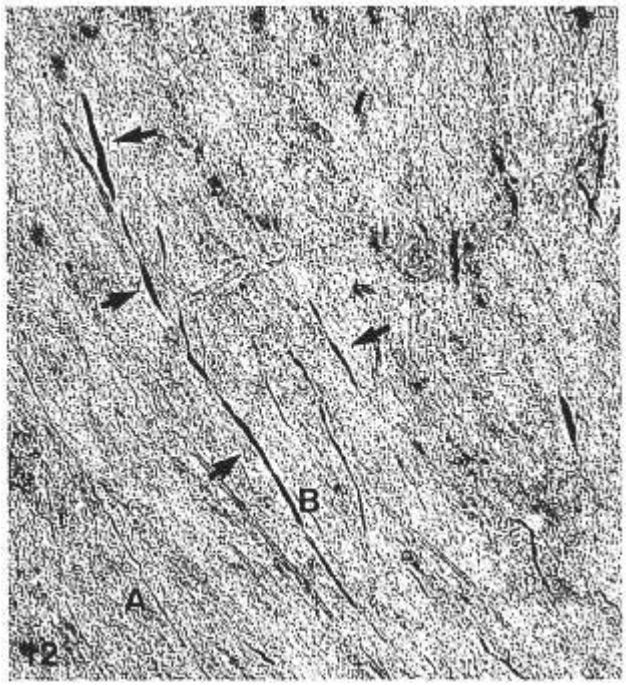

Fig. 12. In this micrograph harvested from the corpus callosum of the control subject, 68 kD immunoreactive axons (arrows) can readily be seen coursing in the field. Note that the immunoreactive axons display a linear profile. Variations in the intra-axonal immunocytochemical reaction reflect the fact that axons course irregularly in the field, moving in and out of the plane of section. Further, note the variation in the diameter of the immunoreactive axonal profiles as evidenced by axons A and B. This most likely reflects differences in axonal diameter. ×200.

impression that those patients sustaining the most severe injury showed proportionately more damaged axons. Interestingly, sites not classically associated with the occurrence of reactive axonal change, including occipital cortices and the basal ganglia, showed evidence of such change in some patients.

Control Case

In the one control case included in this study, no evidence of any of the abnormalities described above was identified (Figs. 12, 13). Again, using the rigorous exclusion criteria detailed in the Materials and Methods section, no evidence of reactive axonal swelling, distention, or separation was seen. Variation in axonal diameter was recognized in the control studies (Fig. 12); yet, this finding was consistent with variations in fiber size typically seen in the brain. Occasionally, axons revealing irregular contour and/or a corkscrew appearance could be identified (Fig. 13). Importantly, however, these intra-axonal changes were not associated with marked distention of the axon cylinder, and as such they appeared quite consistent with those artifactual changes previously associated with the postmortem handling (27). As such potential artifactual

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Fig. 13. This axonal segment, taken from the control case, again demonstrates the presence of 68 kD immunoreactivity. Note that, in this case, a convoluted (arrow) axonal segment can be identified. Although this axonal change is consistent with postmortem artifact due to handling, it is readily distinguishable from the early intra-axonal reactive changes that involve focal axonal distension. ×400.

changes were excluded from our data analysis, we are confident that the reactive changes described are the direct result of trauma and not secondary to any artifactual manipulation of the tissue.

DISCUSSION

This work confirms the usefulness of antibodies targeted to the 68 kD subunit for detecting reactive axonal change in brain-injured humans. The fact that the use of this antibody was not complicated by parallel immunoreactivity in related somata and cell nuclei, in contrast to the SMI 31, SMI 32, and SMI 33 antibodies, contributed to its utility. This approach appears to be useful in the routine pathological setting for two reasons. First, it can be used with vibratomed as well as cryoprotected cryostat sections. Second, immunoreactivity was recognized in all patients, even those in which initial fixation was not accomplished until over 24 h after death. Our limited experience suggests that with increasing intervals beyond 24 h following death immunoreactivity declines, but this factor remains to be critically evaluated.

The chosen immunocytochemical approach has also allowed for a re-evaluation of the sequence of events involved in traumatically induced reactive axonal change. It appears that axonal damage in brain-injured humans does not necessarily involve immediate tearing of the axon. Rather, it appears to involve a focal perturbation of the neurofilamentous structure, which evolves, over time, to culminate in disconnection. Similar to our recently reported findings in experimental animals, these findings in humans suggest that a traumatically induced perturbation of the cytoskeleton is the pivotal event in the pathogenesis of reactive axonal damage (17). In contrast to animals, however, the overall sequence of progression of reactive axonal change appears remarkably slower in humans. This observation is of considerable interest, as it implies that the therapeutic window for intervention in brain-injured humans is longer than that thought to occur in brain-injured animals. Important in our study is the identification of reactive axonal change in head-injured humans within 6 h of the traumatic event. This finding constitutes a departure from the traditional literature, which has long advocated that reactive axonal change cannot be routinely detected unless the patient has survived the traumatic event by more than 12 h (1, 11, 15, 16). Although isolated reports allude to the possibility of the identification of early reactive change (6, 27) these reports have been controversial. Due in part to an incomplete characterization, as well as to pitfalls associated with use of modified impregnation methods, these descriptions of early change have been dismissed by some (28). Some may argue that the reactive changes described in the present communication at 6 h postinjury are subtle, and perhaps open to debate. Yet, we would argue that our rigorous exclusion criteria, which confined analysis to only those axons which could be followed for considerable length in a plane parallel to that of the section, obviated many of the erroneous interpretations that could result if our analysis were expanded to include axonal profiles moving obliquely and/or transversely to the plane of sectioning. More importantly, however, as the reactive changes described at 6 h postinjury in humans parallel reactive changes previously described in experimental animals, we have little doubt that these reactive changes are one and the same. Importantly, in the traumatically brain-injured humans, we have also conducted ultrastructural analyses of the same reactive axonal segments and basically have again confirmed the same repertoire of change seen in experimental animals (unpublished observations). Thus, we feel confident that our early description of reactive axonal change in traumatically brain-injured humans is real. The fact that reactive axons can now be consistently identified within hours of traumatic injury has considerable significance not only in terms of descriptive neuropathology but also in terms of its potential forensic application. We should note that in several cases, traditional silver studies using the Palmgren method were also employed to assess reactive axonal change. Typically, in those patients who survived the traumatic event for at least 24 h, silver-stained reactive swellings could be identified in the same loci demonstrating immunoreactive axonal abnormalities. Importantly, however, in our hands no corresponding silver-stained axonal abnormalities could be identified in those patients surviving less than 24 h (unpublished findings).

Although the results of this investigation demonstrate that antibodies targeted to the neurofilament subunits can detect early reactive axonal change, caution must be exercised in assuming that a specific pattern of axonal change

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must necessarily be equated with a specific time point in posttraumatic survival. For example, it would be erroneous to assume that the isolated identification of a swollen yet non-disconnected axon would necessarily imply that the patient had lived no more than 6–12 h postinjury. As noted in this communication, as well as in our previous studies in animals (17), the sequence of reactive axonal change shows heterogeneity. Specifically, non-disconnected axons can be seen not only in the early posttraumatic period, but also with more prolonged posttraumatic survival (>24 h), when grossly swollen and disconnected axonal segments predominate. Accordingly, one must rely on the predominant form of reactive axonal change seen in the tissue to obtain a reasonable estimate of the duration of posttraumatic survival. Why such heterogeneous axonal responses occur following traumatic brain injury is unclear. Because we observed comparable heterogeneity in experimental animals in which all systemic variables were controlled (17), it would seem unlikely that secondary insults, such as hypoxia and/or hypotension, would be the cause of those variable axonal responses. It is more likely that the diversity of primary traumatic insults in humans accounts for the observed variability of response. Obviously, these issues require further investigation.

Of additional interest in the present communication was the consistent finding of reactive axonal damage throughout the brain. Although some have emphasized the involvement of the corpus callosum, the superior cerebellar peduncle, and the pontomedullary junction as major foci for reactive axonal change (4), the present study confirms data from our laboratory and others suggesting a more global distribution of these reactive events (1, 11, 18, 28). Particularly interesting was the consistent involvement of the cortices at the gray/white interface, as well as the involvement of the basal ganglia. It is obvious that diffuse axonal injury is much more widespread than previously believed and, perhaps, entails more cortical and subcortical involvement than generally recognized. We believe that such widespread axonal damage is more consistent with the myriad of cortical and subcortical neurological findings that plague head-injured patients. This would then suggest that such widespread axotomy is the pathobiological correlate of much patient morbidity.

As noted above, it was also of interest that axons in some cases could be observed to fail at points involving changes in their anatomical course or at foci showing change in tissue density. Although we are uncertain as to the actual pathobiological significance of these observations, one could postulate that axons are predisposed to failure because of differences in their intra- or extracellular environments. Specifically, one could postulate that changes in the axonal cytoskeleton related to a change in axonal course could predispose the axon to failure at that point. Alternatively, one could speculate that the transition from tightly compartmentalized white matter into the diffusely organized gray matter may contribute to local axonal failure. Further study will allow exploration of these important issues.

ACKNOWLEDGMENTS

We would like to thank Mrs. Susan Walker for her outstanding technical assistance and Mrs. Marilyn Bertrand for her efforts in the preparation of this manuscript.

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Received May 5, 1992/Accepted October 6, 1992

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