EXPERIMENTAL NEUROLOGY 126, 291–299 (1994)

Distribution of Forebrain Diffuse Axonal Injury Following Inertial Closed Head Injury in Miniature Swine

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Diffuse axonal injury (DAI) is one of the most frequently encountered types of brain damage resulting from closed head injury. This study was designed to verify whether DAI could be produced in miniature swine by rapid acceleration and deceleration of the head in the coronal plane. Hanford miniature swine (16–19 kg) were anesthetized with 3% isoflurane and their heads accelerated rapidly once through a 60–105° arc in the coronal plane, producing only transient post-traumatic unconsciousness without prolonged coma. All animals made a good recovery and were sacrificed between 6 h and 10 days after injury. The response of forebrain projection systems to this injury was studied using neurofilament immunohistochemistry with antisera to nonphosphorylated (SMI-32) and phosphorylated (SMI-31) epitopes common to heavy (200 kDa) and medium (160 kDa) neurofilament proteins. In 9 of 12 animals, lesions characterized by foci of SMI-32 positive axonal retraction balls were present at the white matter/gray matter junction at the crests of gyri in the dorsolateral regions of the frontal, parietal, and temporal cortices and along margins of the lateral ventricles. A high density of pyramidal neuron perikarya in layers III and V within cortical gyri associated with subcortical DAI were intensely positive for SMI-31 immunohistochemistry. These results validate the use of miniature swine in studies of axonal injury and demonstrate that axonal injury analogous to that seen in the mildest form of DAI (grade I) can be produced in these animals without producing prolonged coma.

INTRODUCTION

Traumatic brain injury in nonhuman primates produced by angular acceleration duplicates many neuropathological and neurological features of human closed head injury (3, 4, 8). Both the degree of lost consciousness and the types of lesions produced in this primate model are a function of the rate, duration, and plane of rotational acceleration. Brief rapid acceleration in the sagittal plane produces concussion and focal lesions including contusion and subdural hematoma, whereas acceleration with longer duration and lower rates, particularly with movement in the coronal plane, is necessary to produce prolonged coma and diffuse axonal injury (3, 8, 10–13). Diffuse axonal injury (DAI) is present in over half of all severely head-injured patients and in more than 85% of vehicular-related severe head injuries. It is responsible for more than one-third of all head injury deaths, for most cases of vegetative survival, and for much of the impairment in survivors of head injury (9). Three grades of DAI are recognized (1, 2, 3). Grade I is characterized by microscopic axonal injury, manifest as axonal terminal clubs and retraction balls, that are present throughout the brain but are especially prominent in the corpus callosum and parasaggital white matter. Grade II DAI is characterized by microscopic foci of axonal injury and macroscopic hemorrhagic tears in the corpus callosum. Grade III DAI is characterized by microscopic and macroscopic lesions in the forebrain as well as macroscopic tissue tears in the dorsolateral brain stem, particularly in the superior cerebellar peduncles. DAI of increasing severity is correlated with progressively longer durations of post-traumatic unconsciousness and increasing degrees of persisting neurological disability (8). The results of inertial closed head injury studies with primates indicate that, although angular acceleration/deceleration in the coronal plane produces a reproducible widespread pattern of axonal injury throughout the brain, the distribution is neither random nor uniform (3, 8, 12, 13). Although axonal injury in the most severe cases of inertial head injury in nonhuman primates is widespread, foci of high-density axonal injury are found within several brain regions (8, 12) and are highly analogous to those seen following human closed head injury (1–4). These foci of high-density axonal injury have been correlated with regions of high tensile strain determined from physical modeling studies (29, 33, 56).

Because tensile strain is directly related to the acceleration rate but inversely related to brain mass, the type of closed head injury that produces DAI cannot be produced by inertial loading in small animals such as cats, ferrets, or rats (11). Due to these constraints, traumatic brain injury has been produced in small

0014-4886/94 $6.00

animals by delivering local mechanical insults to the exposed cortical surface in order to rapidly deform the brain. As a consequence, the damage produced in these models is primarily related to either the contusion produced by impact or to brain stem damage resulting from the movement of tissue down the long axis of the brain, generating levels of strain sufficient to cause axonal injury in regions where the brain stem is extruded through the foramen magna (57). Therefore, the present miniature swine inertial closed head injury studies were undertaken as a first step toward developing a model that could be used to study the mechanisms of diffuse axonal injury and provide a large animal model for testing promising therapeutic agents. The present study was designed to establish whether rotational acceleration/deceleration in the coronal plane could produce DAI in miniature swine.

METHODS

Prior to beginning the animal studies, physical model studies were performed to establish the biomechanical parameters (peak rotational acceleration and peak angular velocity) required to develop strain levels sufficiently high to produce axonal injury (32). Extensive data from studies of similar rotational acceleration/deceleration using human and nonhuman brain/skull physical models (29, 33) facilitated the scaling of these biomechanical parameters for the smaller brain of the miniature swine. As a result of these studies, the number of animals required to validate the model was minimized.

Miniature swine (Hanford strain, males 12–16 kg) were administered an intramuscular injection of the anesthetic ketamine (20–30 mg/kg) combined with the analgesic midazolam (100–500 μg/kg) and supplemented with atropine sulfate (0.05 mg/kg, im) to reduce endobronchial secretions. As soon as the desired depth of surgical anesthesia was attained, characterized by the absence of foot withdrawal response to a mild toe pinch and the absence of the ear twitch reflex in response to a light ear pinch, animals were intubated. Isoflurane anesthesia was initiated at 3% with a flow of 500–600 ml of oxygen per minute until a deep plane of surgical anesthesia was reached at which time isoflurane was cut back to 0.5–1.5%. The right groin area was shaved, prepped with Betadine solution, incised, and the medial saphenous artery cannulated for monitoring arterial pressure and withdrawal of blood samples. The scalp was shaved, prepped with Betadine, and a small incision made over the occipital bone to permit the introduction of a subarachnoid bolt which was screwed into the occipital bone and connected to an intracranial pressure monitor. The incisions were closed, dressed, and the ICP line taped down to prevent disattachment. Noninvasive EKG and EEG electrode leads were affixed to the scalp and chest. The anesthesia level, heart rate, respiration rate, blood pressure, temperature, and EEG activity were continuously monitored and this data collected on a computer-based data acquisition and analysis system. Blood samples were periodically withdrawn to check blood gases.

The animal's head was secured to the injury apparatus by a snout clamp. The lower part of the clamp, a metal plate covered with a rubber bite plate, was inserted into the animal's mouth and the head secured by tightening the padded clamps encircling the snout to the metal plate. Activation of the impulsive loading device rapidly rotated the head through a 60–105° arc in the coronal plane (left to right side motion). The injury, performed only *once* for each animal, took less than 25 ms to complete. Following injury the snout clamp was released, all monitoring lines were checked, and anesthesia was discontinued. Animals remained unconsciousness for periods between 5 and 7 min, as assessed by lack of pinna (mild ear pinch) and eye blink reflexes. Comparable uninjured control animals recovered pinna and eye blink reflexes within 2 min following discontinuation of 1.5% isoflurane anesthesia. Stabilization of cardiovascular and respiratory function and normalization of arterial blood pressure and intracranial pressure typically occurred within the first hour after injury. Following stabilization of vital signs, the animal was extubated, arterial and venous lines were removed, the subarachnoid bolt was disconnected, all incisions were closed, and a topical antibiotic and dressing were applied to the wounds. Upon awakening from anesthesia all animals were administered Buprenorphine (0.1 mg/kg, im) for postoperative analgesia, supplemented every 12 h until sacrifice. During the initial 6 h of postoperative recovery animals were continuously monitored. Once the animals righted themselves, and were able to drink and feed themselves, they were returned to solo cages in the animal colony where most remained for 3–10 days. All procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) and conformed to all USPHS standards for the care and use of experimental animals.

Sacrifice, Histological and Immunohistochemical Processing

Animals were administered a lethal dose of barbiturate, exsanguinated by transcardial perfusion with heparinized saline (1000 units/ml), fixed by perfusion with 3.7% formaldehyde in PBS, and postfixed with 10% sucrose–PBS. Brains were removed and stored in 30% sucrose–saline. The brains were cut coronally in 1-cm blocks for gross examination. Block faces were examined for the presence of contusions, intraparenchymal hemorrhages, or white matter tears, and photographed. Two animals sacrificed with less than 24 h survival were not perfused, their brains were removed, blocked, and immersion fixed in 3.7% formaldehyde in PBS, transferred to

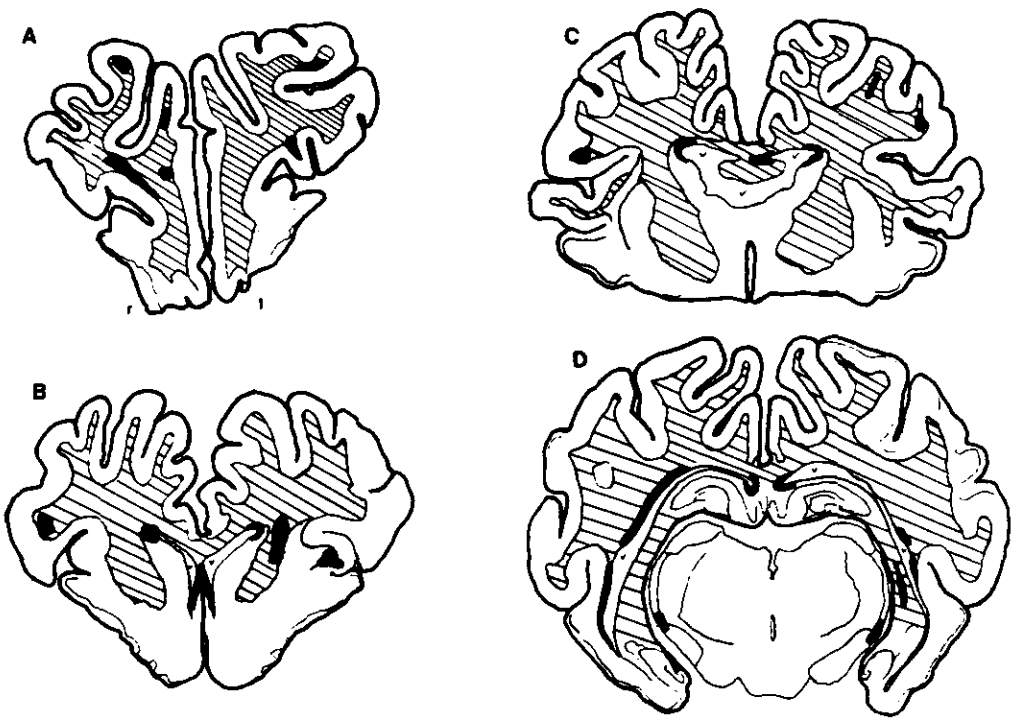

FIG. 1. Distribution of axonal injury (blackened areas) at representative levels of the forebrain of miniature swine following inertial injury in the coronal plane. (A) Prefrontal cortical level. (B) Septal nuclei and anterior commisure level. (C) Rostral-thalamic level. (D) Caudal hippocampal level.

30% sucrose, and subsequently processed similar to the perfusion fixed material. Frozen 40-μm sections were cut from the front face of each block and 10 parallel sets saved. One set was mounted and stained with the Nissl stain cresyl violet. Other sets were reacted for immunohistochemistry using the avidin–biotin peroxidase method (Vector Labs, Ingold, CA). Axonal injury was examined with antibodies that recognize various epitopes on neurofilament proteins: SMI-31 (Sternberger and Meyer Monoclonals), a mouse monoclonal antibody that recognizes epitopes common to phosphorylated heavy (200 kDa, NF-H) and medium (180 kDa, NF-M) neurofilament proteins; SMI-32 (Sternberger and Meyer Monoclonals), a mouse monoclonal antibody that recognizes epitopes common to nonphosphorylated NF-H and NF-M.

RESULTS

To date, coronal plane acceleration/deceleration has been performed on 12 animals. The angular velocity of the coronal plane rotation for the first 3 animals was below the calculated ~270 rads/s threshold for production of axonal injury (Meaney *et al.*, 1993). In these cases, sacrificed between 6 and 24 h after injury, it was difficult to establish whether axonal injury had occurred. In neither case with 6 h survival were retraction balls evident in sections reacted with antisera to nonphosphorylated (SMI-32) or phosphorylated (SMI-31) neurofilaments. In the 24-h case there were some axonal enlargements but these were not so different in appearance from normal fibers as to make a conclusive identification of axonal injury possible.

In all nine cases subjected to angular velocities above 270 radians per second, foci of axonal injury were seen in several brain regions (Fig. 1). Foci of axonal injury characterized by numerous axonal retraction balls labeled by neurofilament immunohistochemistry (Fig. 2A) were found in eight of nine cases. The most common site of this type of axonal injury was at the junction between the white matter and gray matter at the crests of the dorsolateral regions of the frontal, parietal, and temporal cortices (Fig. 2B). In the mildest cases these foci were seen bilaterally in two or three adjacent gyri and in the most severe cases foci were seen in virtually every gyrus between the cingulate and the rhinal fissure. The density of retraction balls within these foci varied between 10 and 100 per 350 × 350-μm grid. Within the foci of axonal injury swollen terminal clubs were present, many of which were attached to a vericose proximal segment (Fig. 2). Based upon the orientation of the proximal segments, it appeared that the damaged axons represented both afferents and efferents of the affected cortical regions. SMI-31 antisera to phosphorylated epitopes common to NF-M and NF-H neurofilament proteins labeled a larger population of more

intensely labeled retraction balls than the SMI-32 antisera to nonphosphorylated NF-M and NF-H protein epitopes.

Axonal injury in the deep cortical white matter, the internal capsule, and cerebral peduncles, was seen in only 4 of 9 cases with injuries above threshold. Neither axonal injury in the parasagittal white matter nor microscopic or macroscopic injury in the corpus callosum were present in any of the 12 cases examined for this study. Individual retraction balls or foci of very low-density axonal injury (<10 retraction balls per 350 × 350-μm grid field) were seen in the caudate-putamen, globus pallidus, dorsal thalamus, and hippocampus (Fig. 3). The hypothalamus appeared devoid of axonal retraction balls.

In every case where DAI was present in the cortical white matter, axonal injury was also present along margins of the lateral ventricles (Fig. 1). The highest density of periventricular axonal injury was seen along the lateral edge of the septum pellucidum, the medial margins of the anterior horn of the lateral ventricle (Fig. 4). At the dorsal and ventral angles of the ventricles along the anterior horn, body, and posterior horns of the lateral ventricles, just lateral to the fasciculus subcallosis, a low to moderate density of axonal retraction balls was present in most cases. Along the lateral margins of the posterior horns of the lateral ventricle a moderate density of axonal retraction balls was present in the tapetum and deep cortical white matter. DAI was not seen along the lateral aspects of the anterior horn of the lateral ventricle at the level of the dorsomedial caudate nucleus or along the lateral margins of the third ventricle in the diencephalon with the exception of the regions of the midline thalamus just above the intraventricular foramen at the caudal end of the intrathalamic adhesion.

One advantage of using SMI-31 immunohistochemis-

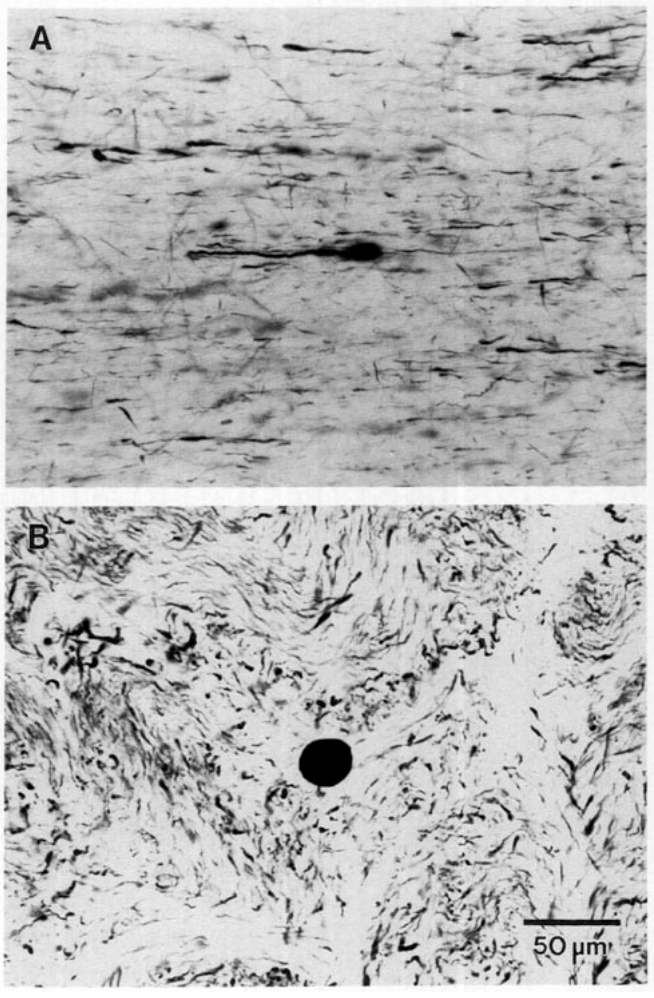

FIG. 2. Axonal injury in cortical gyri following inertial injury in the coronal plane, SMI-31 immunohistochemistry. (A) Retraction balls and axonal swellings in the white matter beneath the middle frontal gyrus. (B) Axonal retraction balls at the white matter/gray matter interface region in the middle frontal gyrus.

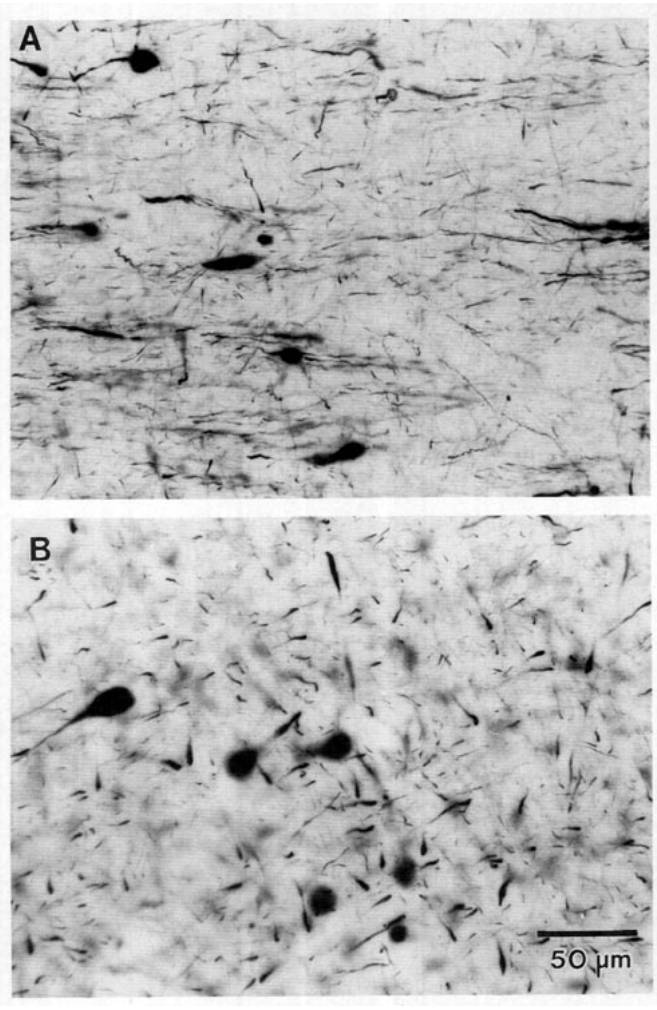

FIG. 3. Axonal injury in the deep white matter following inertial injury in the coronal plane, SMI-31 immunohistochemistry. (A) Axonal retraction balls in the internal capsule lateral to the thalamus. (B) A giant axonal retraction ball in the putamen.

try for the study of axonal injury is that, like other antibodies which recognize phosphorylated neurofilament epitopes normally present only in axons, it also labels perikaya of neurons with damaged axons. Within cortical gyri where DAI was present either at the white matter/gray matter junction or in the deeper subcortical white matter a high density of pyramidal neuron perikarya were intensely labeled, usually in layers III and V (Fig. 5). Moderately labeled pyramidal cell perikarya in layers III and V were also seen in gyri not associated with DAI.

Frank frontal or temporal contusions unrelated to the site of ICP bolt placement were not present in any of the 12 cases examined to date. Burst right olfactory bulbs were present in 2 cases, both of which had experienced basilar skull fractures between the orbit and the cribiform plate. Axonal injury in the ipsilateral lateral olfactory tract, characterized by axonal retraction balls positively labeled with the SMI-31 and SMI-32 neurofilament antisera, was present in both of these cases. Beneath the ICP bolt implantation site in all 10 of 10 cases in which the bolt was in place during the inertial injury, contusions characterized by necrosis of a focal region of cortical gray matter, laceration, axonal injury, and serum extravasation into the parenchyma of the local cortical gray and white matter were present (Fig. 6). An intraparenchymal hemorrhage associated with a deep tissue tear was present ventral to the bolt implantation site in all of these cases. Although the site of bolt placement had been varied from frontal to parietal to occipital and left to right, similar intraparenchymal hemorrhages were found in every case. In some cases these tears were continuous with the cortical surface but in most cases the hemorrhagic tear was between 5 and 15 mm ventral to the bolt implantation site. These lacerations and deep intraparenchymal hemorrhages did not represent the track of the bolt through the cortical parenchyma as the bolts were placed and remained superficial, penetrating at most only 2–3 mm into the hemispheres. Axonal injury, characterized by retraction balls positively labeled with neurofilament immunohistochemistry, was present along the margins of the hemorrhagic tear and diffusely throughout surrounding gray and white matter areas (Fig. 6). Foci of axonal retraction balls labeled with neurofilament immunohistochemistry were identifiable proximal to the probe implantation site in the case with 12 h survival but not at the periventricular or cortical white matter/gray matter junction regions where foci of axonal injury are regularly seen in cases with longer survival times. Minimal neuronal loss was evident in cortical regions surrounding the bolt implantation site, generally limited to zones of complete neuronal loss that were either

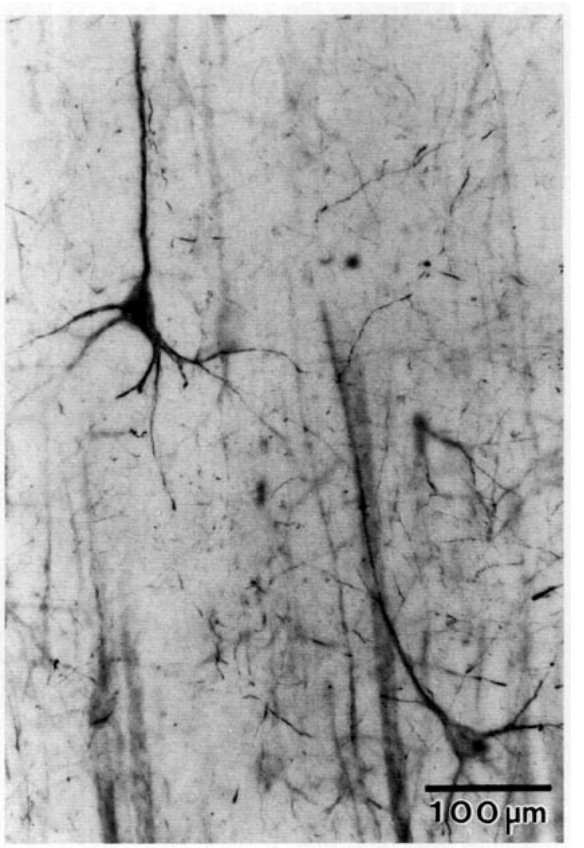

FIG. 5. Phosphorylation of perikaryal neurofilaments in neocortical pyramidal neurons in layer III of the middle frontal gyrus following inertial injury in the coronal plane, SMI-31 immunohistochemistry.

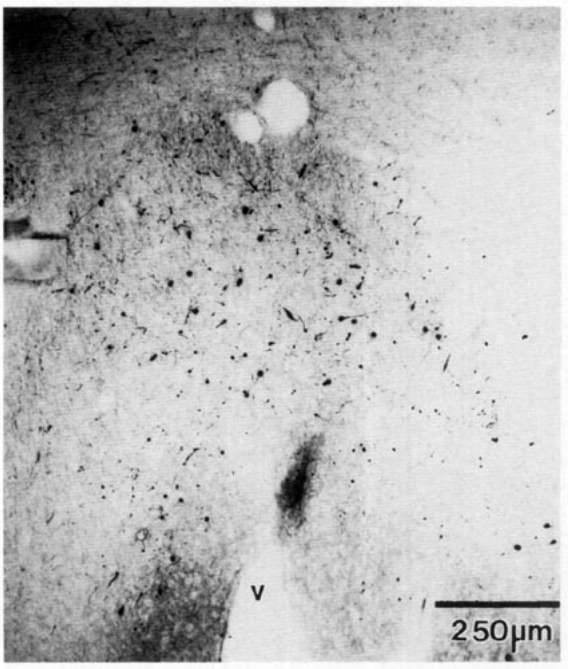

FIG. 4. Periventricular axonal injury following inertial injury in the coronal plane, SMI-31 immunohistochemistry. A high density of retraction balls are found at the dorsal margins of the anterior lateral ventricle.

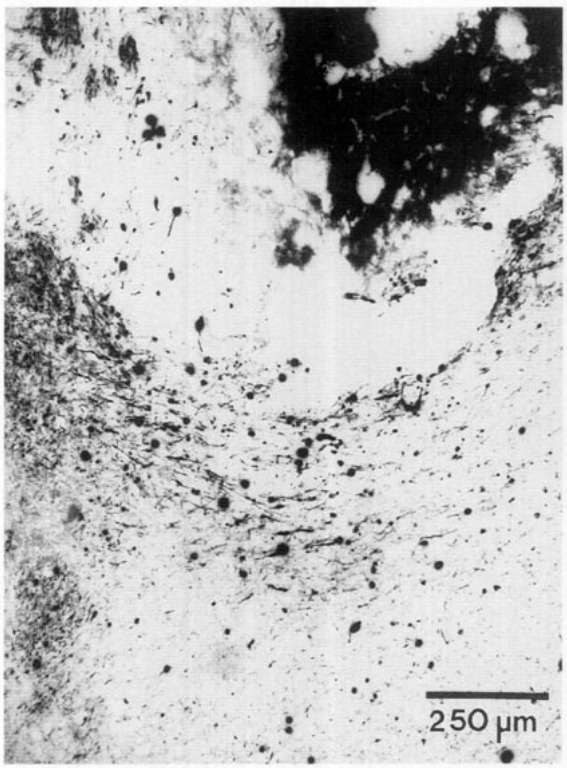

FIG. 6. Axonal injury adjacent to tear associated with ICP bolt implantation 3 days following inertial injury in the coronal plane, SMI-31 immunohistochemistry. A high density of axonal retraction balls are present at the ventral margin of a hemorrhagic cavity in the putamen 7–10 mm ventral to the site of ICP bolt placement.

only partial depth (superficial) or narrow 1–2-mm wide bands adjacent to some margins of the bolt lesion. In the two cases with subdural hemorrhages more extensive (4–8 mm wide) regions of complete neuronal loss were seen centered on the bolt placement site. In both of these cases extensive neocortical regions were covered by the subdural blood clot but appeared completely normal. Extravasation of serum proteins, identified by immunohistochemical visualization of pig IgGs, was evident throughout the gray matter associated with contusions at the bolt implantation site and radiating out from the contusion site through the white matter for some 4–5 mm.

DISCUSSION

Axonal damage has been classically demonstrated using reduced silver techniques (42) and observations based upon these techniques make up the bulk of the description of axonal damage following human head injury (1–5, 37–39, 50, 55). From a study of DAI in inertially injured primates we established that SMI-31 immunohistochemistry labeled populations of axonal retraction balls and varicose axonal swellings comparable to those labeled with reduced silver labeling techniques (45). The present study verifies that in miniature swine, as in nonhuman primates and humans, SMI-31 and SMI-32 immunohistochemistry are excellent markers of axonal injury which selectively label features of axonal pathology. In the normal CNS it is well established that antisera which recognize phosphorylated epitopes of the heavy (NF-H) and medium (NF-M) protein subunits, such as SMI-31, label axonal neurofilaments, and that antisera which recognize nonphosphorylated NF-H and NF-M epitopes, such as SMI-32, label perikaryal and dendritic neurofilaments (26, 52, 54). It is our experience that in large animals, including swine, nonhuman primates, and man, antisera to phosphorylated epitopes of heavy and medium neurofilament proteins (SMI-31) label more retraction balls and label them more intensely than SMI-32, the comparable antisera for nonphosphorylated NF-H and NF-M (45). We have found the reverse to be true in studies of axonal injury produced by cortical impact (46, 47) and fluid percussion injury in rats (44) where SMI-32 labeling is very dense and intense and SMI-31 labeling of retraction balls is much more faint and selective. Our results confirm the conclusion of Povlishock (40) that neurofilament immunohistochemistry is a powerful tool for the identification of damaged axons in head injury models and extends them by establishing that the well-characterized antisera SMI-31 and SMI-32 (54) are excellent markers for axonal and perikaryal injury following head injury.

Axonal injury not associated with ICP bolt placement was found in 8 of 12 miniature swine subjected to acceleration/deceleration in the coronal plane. These microscopic lesions are analogous to those seen in the mildest form of diffuse axonal injury (grade I) in nonhuman primates subjected to similar nonimpact rotational acceleration. The anatomical distribution of these foci of axonal injury in miniature swine was different from that seen in primates (3, 8). Whereas in primates with grade I DAI foci of axonal retraction balls were most frequently found in the parasagittal white matter and corpus callosum, these locations were virtually devoid of axonal retraction balls in miniature swine. Instead, axonal injury in miniature swine was found predominantly at the white matter/gray matter junction in the dorsolateral gyri of the frontal and parietal lobes and around the margins of the lateral ventricles. Indications of more severe grades of DAI, characterized in nonhuman primates and human head injury victims by focal hemorrhagic tears in the corpus callosum (grade 2), and in the most severe cases by hemorrhagic tears in the rostrolateral brain stem at the level of the superior cerebellar peduncles (grade 3) were absent in the brains of inertially injured miniature swine examined in this study.

What accounts for the different distribution of DAI in miniature swine and primates? The gross anatomy of the porcine brain and cranial vault is different from the

primate brain and skull. Most noticeably different are the pig's relatively large olfactory bulbs that drop almost vertically off of their thin and elongated frontal lobes, their relatively small temporal lobes, the virtual absence of a falx, and their brain stem and spinal cord which, as in all quadrupeds, extend in continuity with the long axis of their forebrain. As a consequence of these structural differences the distribution of strain due to angular acceleration/deceleration of the miniature swine brain in the coronal plane (32) is different than the distribution of strain produced by similar angular acceleration/deceleration in primates (29, 33). The absence of axonal injury in midline structures, particularly the corpus callosum and parasagittal white matter, together with the high incidence of injury in the middle frontal gyrus and other more lateral regions of the cerebral hemispheres may reflect different patterns of brain displacement that occur in the presence of a falx that extends only superficially between the hemispheres (32). Frank frontal or temporal contusions which occur with high frequency in human head injury as well as experimental primate inertial injury (3) have not been produced by coronal plane rotational acceleration/deceleration in miniature swine. The reason for the lack of contusions in this model are unclear.

The medial and lateral margins of the lateral ventricles were also frequent foci of axonal injury following inertial injury to miniature swine. Periventricular axonal injury is not unique to the miniature swine model but its ease in detection using neurofilament antisera reflects the higher technical sensitivity afforded by immunohistochemistry. Periventricular distribution of axonal injury has been described in human closed head injury (18, 19). In the brains of primates subjected to lateral inertial head injury periventricular axonal injury is much more evident in sections reacted for neurofilament immunohistochemistry, particularly SMI-32, than in adjacent silver stained sections, due to the relatively high amount of background labeling and the nuclear labeling present in silver stains (45). Whether the mechanism of periventricular axonal injury reflects the transient (millisecond) massive increase in intracranial pressure thought to occur immediately following lateral acceleration/deceleration (Thibault, personal communication), causing volumetric expansion of the lateral ventricles that places fibers coursing near the sharply angled ventricular margins under higher levels of strain, or whether high strain levels result from deformation in these regions due to the inertial movement itself are at present unknown but are the subject of planned physical modeling studies.

Perikaryal neurofilament phosphorylation has been documented in many species following injury of numerous neuronal systems (7, 16, 24, 25, 28, 43, 49). We have observed neocortical pyramidal neuron perikaryal neurofilament phosphorylation in man and nonhuman primates following head injury (45), and following fluid percussion or cortical impact injury in rats (44, 46, 47) using SMI-31 immunohistochemistry. The severity of injury required to induce perikaryal neurofilament phosphorylation is not well established. It has been suggested that expression of perikaryal pNFs following axonal injury may require injury proximal to the cell body and that injury more distal on an axon may either fail to induce perikaryal neurofilament phosphorylation or induce only barely detectable levels in affected perikarya (28). Conversely, direct dendritic or perikaryal injury may be more likely to induce a more rapid and extensive perikaryal neurofilament phosphorylation.

Perikaryal neurofilament phosphorylation has also been reported to occur following administration of agents that disrupt axonal transport (14, 15) and in numerous human neurodegenerative diseases (20, 22, 23, 34, 35, 41, 51, 58). Perikaryal neurofilament phosphorylation also occurs in regions adjacent to focal infarcts in human brain (27) and in motoneurons of the oculomotor nuclei and spinal cord anterior horn (48) and cerebellar Purkinje cells (6) following hypothermic cardiac bypass and arrest in neonatal swine. Perikaryal neurofilament phosphorylation has been postulated to reflect an adaptive response that protects neurofilaments from proteolysis (17), but the maladaptive consequences of abnormal neurofilament phosphorylation may contribute to subsequent neuronal death or persisting perikaryal abnormalities (36).

Although the pattern of axonal injury in miniature swine is not identical to that seen following similar coronal plane acceleration/deceleration of the head in primates, the nature of axonal injury appears highly analogous to that seen in type I DAI. Whereas in the nonhuman primate model axonal injury and coma were highly related (8), the fact that it is possible to produce substantial axonal injury in miniature swine without producing prolonged coma makes the miniature swine model very attractive both for the study of the cellular mechanisms involved in secondary axonal injury and for use in preclinical trials of therapeutic agents that interrupt its pathophysiological process.

ACKNOWLEDGMENTS

This work was supported by the NINDS Head Injury Center Grant NS-08803-21-22. The authors acknowledge the assistance of Leonardo Rodriguez-Cruz, M.D. and Ms. Beth Ann Moore in surgical preparation of the animals; Jane Brasko, Ph.D., Ms. Claire Coggins, and Ms. Hahnah Kasowski for histological and immunohistochemical processing and darkroom work.

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