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Original papers

Neural tube defects

Some remarks on the possible role of glycosaminoglycans in the genesis of the dysraphic state, the anomaly in the configuration of the posterior cranial fossa, and hydrocephalus

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Abstract. Recent developments in the field of experimentally induced neural tube defects (NTD) indicate that specific substances, namely the glycosaminoglycans (GAGs) may play a role in the genesis of spinal malformations. The authors report the results obtained by evaluating the GAGs in rat fetuses with NTD, secondary to the administration of Trypan Blue during pregnancy. A characteristic decrease in GAGs formation in the spinal and cranial structures as well as in the subependymal regions of the brain was found in the malformed fetuses. The authors hypothesize that this anomaly in GAGs formation is responsible for both the NTD and the associated malformations, namely hydrocephalus and hypoplasia of the posterior cranial fossa.

Key words: Neural tube defects – Trypan Blue – Glycosaminoglycans – Hydrocephalus – Meningocelemyelo – Chiari malformation.

In spite of the more refined techniques of experimental investigation and clinical research, the pathogenetic theories of neural tube defects (NTD) are still deeply influenced by the early pathogenetic interpretations, namely the hypothesis of the primary lack of fusion of the neural tube, and that of the secondary rupture of a previously fused neural tube.

As most of the studies have focused on the problem of the spinal defect, the anomalies associated with the NTD, namely hydrocephalus and posterior cervical fossa hypoplasia, have almost always been regarded as secondary to the spinal malformation and explained by postulating mechanical causes.

The aim of the present paper is to describe some experimental findings which, in our opinion, allow interpretation of both NTD and the associated anomalies of ectodermic and mesodermic structures.

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Historical background

The first anatomical description of spina bifida was given by Tulpius [58], who in 1652 recognized the pathological presence of a nervous mass between opened spinal arches, a condition which he defined "spina dorsi bifida."

Morgagni [30] was the first, in 1761, to describe the correlation between hydrocephalus and spina bifida and to propose a unique pathogenetic mechanism to explain the association of these two conditions. He suggested that dysraphism could result from dilatation caused by fluid accumulation (*"hydrops cerebri et medullaris"*) and the consequent rupture of a previously closed neural tube.

The "dropsy" theory and secondary rupture of the neural tube was still considered by Cleland in his paper "Contribution of the study of spina bifida, encephalocele and anencephalus", published in 1883 [6], together with a second hypothesis, i.e., deficient closure of the canal of the cerebrospinal axis or its coverings. The latter theory had obviously been influenced by the studies of Saint-Hilaire [44] on the influence of environmental and genetic factors on the embryos' development, which some years later, in 1886, would lead F. von Recklinghausen to suggest "a primary defect" of neurulation as the cause of the non-closure of the neural tube [39].

In this century, two main pathogenetic theories have been propounded. The first of these still considers the lack of fusion of the neural tube as the first step leading to spina bifida. Variations on this theory have been proposed by several authors. In fact, Dekaben [8] indicated that the primary defect lay in an "abnormal orientation and organization" of the cells of the neural folds.

Recently, Linville et al. [23] drew attention to the cytoskeletal system, and especially the microtubule-microfilament system, inhibition of which can disturb the normal elevation and apposition of the neural folds to form the neural groove and then the neural tube [16]. This hypothesis has been confirmed by several experimental reports and anatomopathological researches in human dysraphic fetuses, and it is one of the most widely accepted at the present time.

Marin-Padilla [26, 27] suggested that the non-closure can also arise from a defect in the mesoderm rather than in the neuroectoderm. This author pointed out a "primary mesodermal insufficiency" with a reduction in the number of available paraxial mesodermal cells. However, several experiments showed that the formation of the neural tube does not depend on external forces, such as the mesoderm, since the neural plate, or parts of it, either isolated or transplantated, can close to form a neural tube [1, 4, 14].

Patten [38] described an abnormal neural tube proliferation in several embryos, some of which had no external defects. He proposed that a local "overgrowth" of the neural plate might prevent its closure. However, Dekaben [8] found no deficient or excess nucleus counts in the anencephalic neural fold tissues, and Lendon [21], by using an autoradiographic technique in normal rats and in embryos with early Trypan-Blue-induced spina bifida, found scarce statistical difference between normal and abnormal embryos. Obviously, the observations of the last two authors do not support the "overgrowth/non-closure" hypothesis.

The second theory, which derives from Morgagni's rupture hypothesis, was originally proposed by Gardner [11] and Padget [37] in the late 1960s, and recently by McLone et al. [28]. Gardner reported the anatomical observation of the impermeability of the roof of the IV ventricle to the cerebral fluid, resulting in a subsequent increase in the intra-tube pressure and its rupture. This increase is responsible, according to Gardner, for hydrocephalus, encephalocele, hydromyelia, syringomyelocele and Chiari malformation. Further support for the idea of reopening of the neural tube has been reported by Keen [17]. He postulated that this pressure increase may result from several factors: abnormal secretion of the choroid plexus, blockage of a foramen in the roof of the hind brain, interference with the absorption of the fluid. It is important to consider that this hypothesis shifts the time of pathogenesis to a later period of embryological development with respect to the non-closure hypothesis.

However, there is also a great deal of evidence against the rupture theory: no abnormalities were found in the histological features of the roof of the IV ventricle or in the primitive subarachnoid space of human dysraphic embryos [35]; myeloschisis was reported in many early embryos before stages 17 and 18, which is when CSF begins to flow out from the IV ventricle [34, 47, 59]; a normal permeability of the roof of the IV ventricle to CSF was reported even in cases with no anatomical evidence of foramina [7]; there was no high incidence of hydromyelia, syringomyelia or spina bifida in animals in which the foramen of Magendie is normally absent [7].

More recently Lemire has suggested a new theory. The most caudal part of the spinal cord derives from a recanalization of the caudal undifferentiated cellular mass, at least in the chick [43] and in the rat [2], and perhaps in human [20].

On the grounds of this embryological observation, Lemire [20] suggests that some neural malformations in the lower lumbar and sacral regions might result from an abnormal development and recanalization process of the tail-bud material. Osaka et al. claim that this is possible for meningocelemyelo [35].

Materials and methods

Experimental induction of hydrocephalus

Wistar rats weighing 200–250 g were used for this study. They were housed under conventional conditions with free access to food and water.

Various groups of rats were mated from 4 p.m. to 8 a.m., and during that time observation of the vaginal mucous plug established day 0 of pregnancy. A total of 20 pregnant rats was used. Subcutaneous injections of 2 ml of a 4% saline solution of Trypan Blue (B.D.H. stain, 34078) were administered to 15 of these in the scapular region between 11 and 12 a.m. each day from the 7th to the 9th day of pregnancy. The remaining 5 rats were used as controls and received injections of 2 ml of saline solution according to the same administration schedule.

Study of the skeletal system

For the evaluation of the skeletal system, 10 fetuses removed from the maternal membranes were immediately fixed in 96% ethanol. After 2–3 h the skin and the internal organs were removed with the aid of a dissecting microscope, and fetuses were then left in 96% ethanol for 24 h.

Afterwards, the fetuses were immersed for 24 h in xylene, and then stained in a fresh daily solution of 96% ethanol (80 ml), acetic acid (20 ml), Alcian Blue (15 mg; G.T. Gurr) for 3 days and subsequently dehydrated for 5 days in 100% ethanol. The complete clearing of the soft tissues was finally accomplished by leaving the fetuses immersed in a 2% solution of KOH for 3–4 days. At the end of this procedure the cartilaginous structures were stained in blue.

In order to colour the osseous structures the fetuses were stained in a 0.002% solution of Alizarin Red (Merck stain) in 2% KOH for 24 h. The excess red stain was eliminated by repeated daily washings, first in Mall's solution (79 parts of bidistilled water, 20 parts of glycerin, 1 part of 0.7% solution of KOH) and then in progressively concentrated (25%, 50% and 80%) solutions of glycerin. The fetuses treated in this way were stored in 100% glycerin [41].

Examination of glycosaminoglycans (GAGs)

The fetuses not used for the study of the skeletal system were fixed in Bouin's solution for 3 days, with daily changes of fixative. The fixative was always prepared just before use.

After several washings in 70% ethanol, serial coronal sections were made of the head of the fetal rats according to Wilson's technique [62].

The various sections were then dehydratated and embedded in paraffin blocks from which 2.0-μm-thick sections were obtained. After removal of the paraffin and rehydration the sections were divided into two groups and left to react separately for 10 min in two solutions of 0.2% Alcian Blue in acetic acid at pH 2.5 or 1.0 [22]. As these solutions are not stable, they were prepared and filtered shortly before use. The sections were then treated for 15 min with three successive washings with bidistilled water, then immersed in a 2% solution of acetic acid for 5 min. The sections were finally counterstained with Carminic Acid, dehydrated, cleared, and mounted in Eukit.

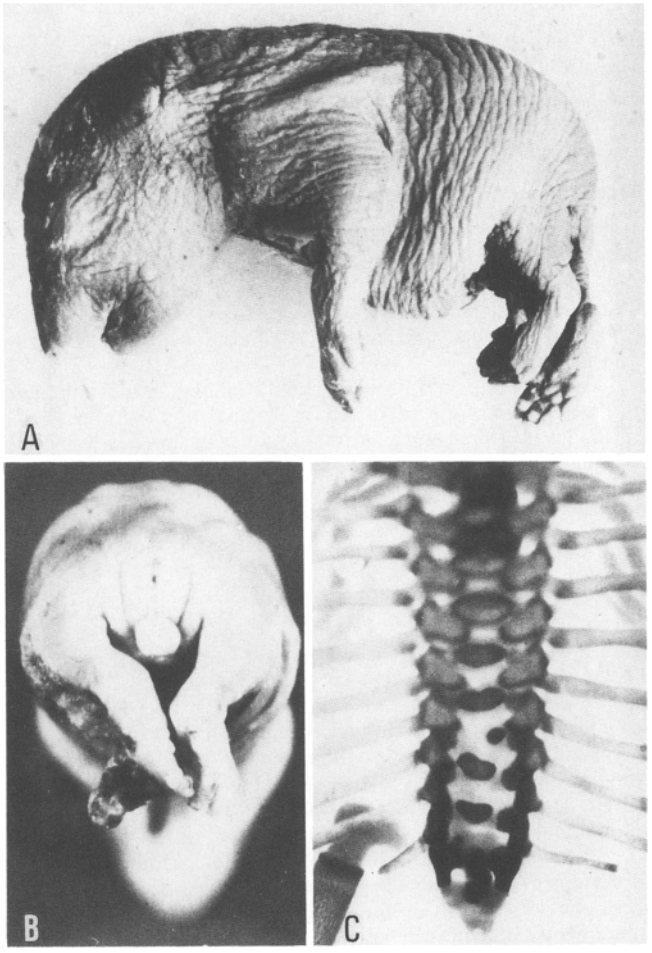

Fig. 1 A–C. Twenty-day-old fetus with neural tube defect and hydrocephalus: note increased head size and hypoplasia and weakness of the posterior limb (**A**) due to a neural tube fusion failure in the lumbosacral region (**B**). Alcian Blue-Alizarin Red staining revealed abnormalities of the last thoracic and lumbosacral vertebrae (**C**)

Results

A total of 90 live fetuses was obtained from the Trypan Blue group, 6 of which (6.6%) turned out to be hydrocephalic; in 3 of these there was an obvious dysraphic state of the spine (Fig. 1). In the control group there was a total of 40 live fetuses. All were normal.

Skeletal system

Examination of the skeletal system using the double staining method revealed anomalies in the configuration of the skull base of the hydrocephalic fetuses compared with the control animals.

Splitting of the cranial sutures was associated with underlying hydrocephalus (Fig. 2). The skull base appeared to be relatively elongated, with a flattening out of the angles between the basi-sphenoid and the basi-occiput and between the basi-occiput and the cervical vertebral canal (Fig. 3).

Fig. 2. Skull of a 20-day-old rat fetus with hydrocephalus: note diastasis of the cranial sutures. (Alcian Blue-Alizarin Red)

The overall dimensions of the posterior fossa were reduced in malformed fetuses, but the diameter of the foramen magnum and the atlas was increased (Fig. 4).

GAGs evaluation

In normal animals, the GAGs appeared as a thin blue film over the ependyma and the surface of the choroid plexus. GAGs material was also seen within the cerebral ventricles, especially in relation to the choroidal plexus surface facing the ventricular wall.

In all the hydrocephalic fetuses, the ependyma was not covered by GAGs and spongiotic changes were observed in the subependymal structures (Fig. 5). Also, the choroid plexuses did not show GAGs staining. It is worth noting that an overall decrease in GAGs content, including bone and skin tissues, was noted in the hydrocephalic animals.

Similar results were obtained with staining techniques at pH 1.0 and 2.5; the only difference recorded was a more intense coloration when the Alcian Blue stain was used in pH 2.5 solution.

Discussion

Despite the enormous amount of experimental and clinical research, the aetiology of NTD and their associated malformations remains quite unclear [40]. Indeed, the study of NTD offers several difficult points for discussion, such as the nature of the mutual relationship between the forthcoming neural tube and the axial skeleton, the importance and the function of the embryonal extracellular matrix in the development of the neuroectodermal and mesodermal derivatives, and the origin of the associated hydrocephalus and cerebral tissue malformations.

The use of the experimental models of NTD and the recent studies in the CNS embryology have lent further support to the opinion that the non-closure hypothesis is the most probable explanation of the origin of the dysraphic states [33]. Lemire's recent theory, which presents

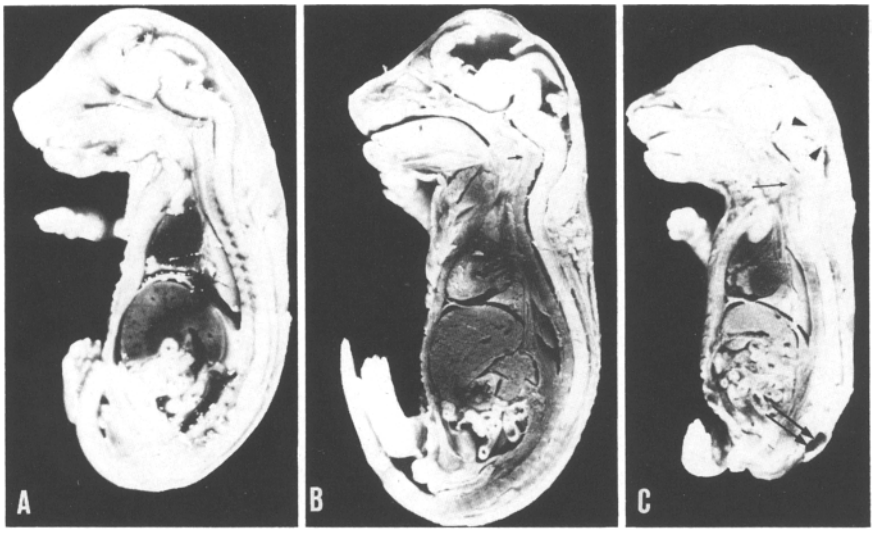

Fig. 3A–C. Twenty-day-old rat fetuses. **A** Normal fetus; **B** hydrocephalic fetus; **C** fetus with myelomeningocele (*double long arrows*). While the fetus with primary hydrocephalus (**B**) maintains anormal basi-occiput angle (*short arrow*), the fetus with myelomeningocele (**C**) shows a flattened angle (*long arrow*) with caudal dislocation of the brainstem and cerebellar structures (*head arrows*)

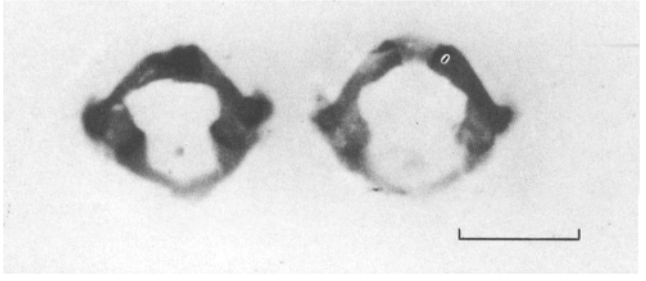

Fig. 4. Comparative size of Atlas in a normal 20-day-old rat fetus (*left*) and in a fetus of the same age with Chiari type II malformation (*right*). Note increased diameter in the abnormal fetus. (Alcian Blue-Alizarin Red)

some stimulating aspects, still has to be checked in humans.

Probably the most interesting observation yielded by experimental studies of NTD is that several chemical or physical agents can induce this type of anomaly, acting through different mechanisms [9, 40].

Consequently, the multifactorial aetiology of spina bifida and the concept of gene-environmental interaction are widely accepted at present. Theories pointing to a single cause, e.g. cytochalasins from potato intake, or increased body temperature resulting from sauna or illness, must now be definitively rejected. The study of the neural tube/axial skeleton relationship appears to us to offer an important insight into the genesis of NTD.

Correct development of the spinal cord is in fact necessary for normal development of the axial skeleton. This relationship has been demonstrated by the following experimental observations. Lipton and Jacobson [24] and Packard and Jacobson [36] have demonstrated that the neural plate imposes the segmentation on the presomitic mesoderm and that the somites do not form simultaneously but in an antero-posterior sequence, just behind the advancing notochord. Minor [29] has also found that the cranial somites organize themselves into dermatomyotome and sclerotome whilst the caudal somites are still segmenting. The importance of the spinal ganglia and notochord in the formation and correct segmentation of the vertebrae is well demonstrated. Spinal ganglia seem to control the segmentation of the neural arches, while the notochord seems to control the segmentation of the vertebral bodies [49, 50]. Furthermore, the spinal cord also plays a role in remodelling the vertebral column, thereby preventing compression of the developing spinal cord [19]. This could explain the increased diameter of the atlas in Chiari malformation. The influence of the spinal cord on the axial skeleton formation has also been demonstrated in vitro by inverting the spinal cord of embryonic Urodeles. The axial skeleton which subsequently developed was also inverted [15].

These experimental data could indicate that an intact and morphologically normal neural tube is necessary to induce a normal axial skeleton. Thus, an altered neural tube alone could lead to an altered axial skeleton.

A second mechanism which should also be considered is the action exerted by the extracellular matrix (ECM). In fact, several experimental observations besides the findings provided in the present study seem to indicate a specific role of an altered ECM in inducing NTD. The GAGs are the characteristic component of this ECM, forming "an easily penetrable extracellular matrix through which neuronal migration and differentiation may take place during brain development" [25].

In the development of the central nervous system it has been demonstrated how first the neural tube and the notochord, and later the somites, synthesize a typical matrix rich in GAGs (especially hyaluronate and chondroitin sulphate), in which the cells of the sclerotome migrate to form the vertebrae, the intervertebral discs and the chondral portion of the occipital bone. Using ultrastructural, autoradiographic and histochemical techniques. Specific distributions of GAGs have been found in the

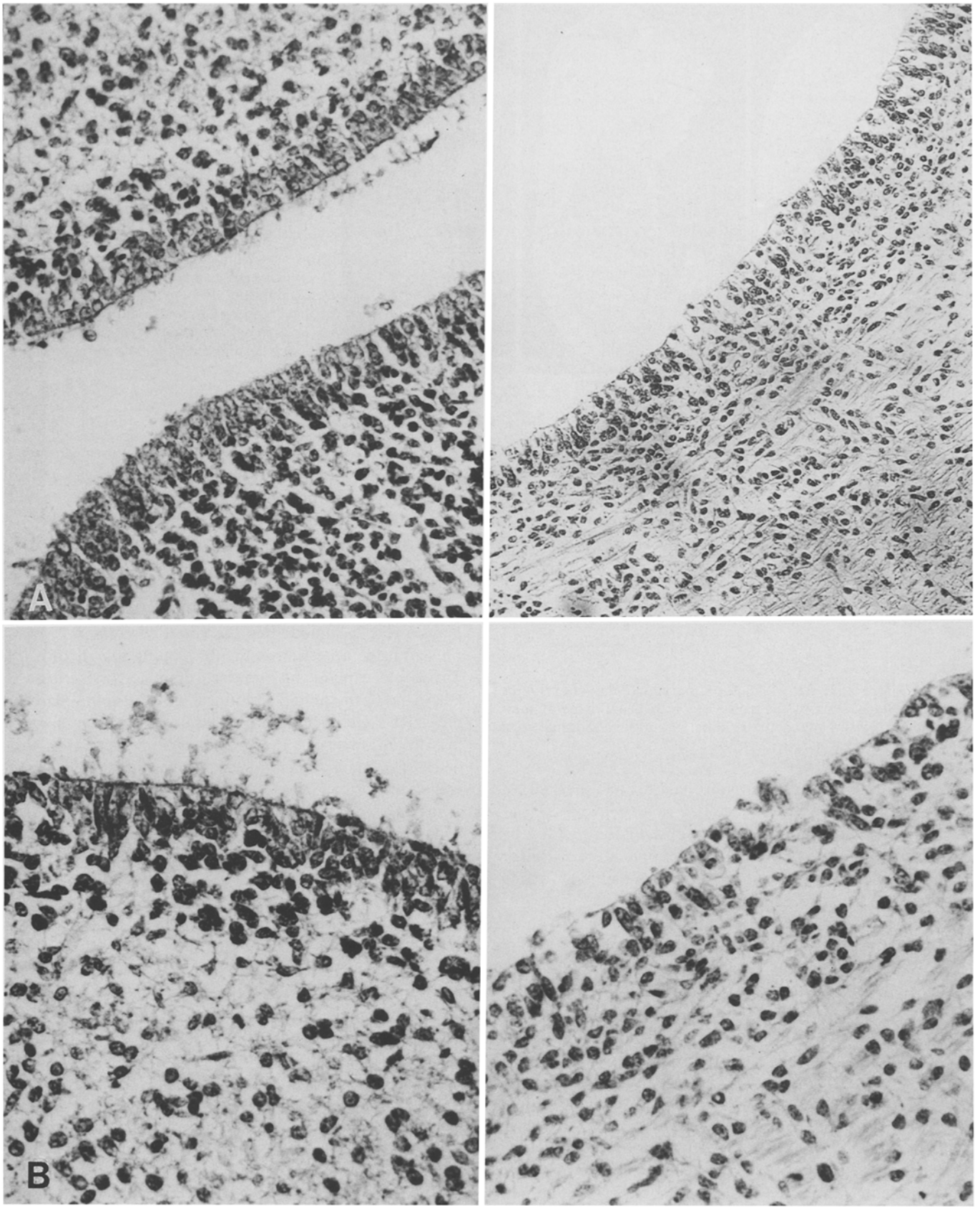

Fig. 5 A, B. Twenty-day-old rat fetuses. **A** *Left,* ependymal surface in a normal rat fetus. Note thin dark film over the ependymal surface corresponding to the presence of GAGs (×400); *right,* ependymal surface in a rat fetus with hydrocephalus and spina bifida. Note absence of dark staining over the ependyma and associated spongiosis of the subependymal structures (×250). **B** Higher magnification of an ependymal surface in a normal rat fetus (*left,* ×600) and in a rat fetus with hydrocephalus and spina bifida (*right,* ×600). Alcian Blue at 2.5 pH

ECM before and after the migration of the neural crest cells [13].

The dispersion of these pluripotential neural crest cells is not random, but rather temporally and spatially ordered under the influence of this local environment [5, 31, 32, 61].

Furthermore, other experiments have revealed a striking vimentin-GFA transition occurring during glial differentiation, with the consequent possibility that immature vimentin-positive neuroglia would display mesenchymal properties, such as the production of this ECM [3].

The importance of the environment on the chondrogenesis has been also demonstrated by using an in vitro system of somites and neural tubes obtained from chick embryos. In fact, the addition of GAGs to the medium of such systems causes a significant increase in the amount of cartilage produced [18]. On the other hand, the addition of enzymes, such as hyaluronidase and collagenase, which degrade the matrix during the early stage of development, induces the inhibition of chondrogenesis; this may occur again after transferring the somite and the neural tube to a medium devoid of the above-mentioned enzymes [51–54]. The inhibition of the differentiation of cartilage has, therefore, been associated with the damage to the extracellular matrix around the notochord and the neural tube. It should be pointed out that hyaluronidase has proved an effective agent in inducing neural tube defects in experimental animals [45].

The invasive ability of the neural crest cells is accompanied by an ability to destroy some components of the ECM. Toole [56] and Weston [60] have reported that at about the time of the migration of the neural crest cells there is an increase in hyaluronidase activity and a decrease in hyaluronate. Weston also showed that enzymatic removal of hyaluronate prevents the migration of the neural crest cells from the mesencephalic neural folds. Toole [55] found that if hyaluronate was administered to cartilage cells in vitro they were induced to migrate. If hyaluronate was removed by using hyaluronidase, migration was inhibited and the cells underwent cytodifferentiation. Hyaluronidases have also been reported to play a controlling role in the cell division cycle, in gene expression [46], and in controlling the permeability of tissues and in the regulation of the ionic environment within the embryo [48].

The experimental results described above clearly indicate that NTD might result from an alteration of the neutral tube/axial skeleton relationship, from an alteration of the embryonic ECM, or from a combination of both, which explains the wide range of the phenotypic presentation of NTD. In fact, the features of the malformation can vary according to whether an agent acts on the neural tube or on the ECM and also with the time and intensity of exposure.

A further aspect to be considered is the almost constant association of NTD and hydrocephalus. The findings obtained with our experimental model are comparable to human pathology, thus confirming the validity of the experimental models in the study of the aetiopathogenesis of NTD.

The same teratogenic effect of Trypan Blue, the agent we used, is also presumed to occur by modifying the GAGs formation through an increase in glycogen production and a decrease in the extracellular synthesis of chondroitin sulphate [12]. In half of our hydrocephalic fetuses the ventricular dilatation was associated with spina bifida. Marin-Padilla [27] has postulated that the Chiari malformation accompanying experimentally induced spina bifida obtained by oral administration of vitamin A, is due to a mesoderm insufficiency resulting in a short posterior cranial fossa. Vitamin A also seems to act by altering the extracellular substrate [10].

Besides the pathogenesis of NTD, GAGs could be also involved in the origin of the hydrocephalus that often accompanies a dysraphic status. Richardson [42], in his study of a strain of mice with recessively inherited hydrocephalus, demonstrated an overall deficit of GAGs. He postulated that a genetic defect was responsible for the alteration in GAGs formation and, consequently, in the chondrification. Richardson explained the hydrocephalus as due to the subsequently altered basi-occipital bone, responsible in turn for the limited development of the posterior fossa subarachnoid spaces. The author did not test the ependyma and cerebral parenchyma for GAGs, however.

Torack et al. [57] used histochemical methods and demonstrated the presence of two families of GAGs in the subependymal region of the lateral cerebral ventricles. The first of these was formed by chondroitin sulphate which reacts with Alcian Blue at pH 1.0, and the second by carboxylic GAGs which react with Alcian Blue at pH 2.5 [22]. The authors hypothesized a possible functional role of these two families of GAGs in the diffusion of fluid between the ventricles and the brain parenchyma, as well as in the production of cerebrospinal fluid. The results we obtained seem to confirm the importance of GAGs in the exchange of fluid within the brain. In fact, the absence of GAGs over the ependymal surface and the choroid plexuses was constantly accompanied by obvious hydrocephalus in our animals. However, in our experiments the alteration in GAGs formation induced by the administration of Trypan Blue induced similar anomalies in the configuration of the posterior cranial fossa to those that occur spontaneously in genetically hydrocephalic mice.

Thus, analogous results can be obtained in two absolutely different situations: in a genetic defect and in a chemically induced alteration, which have in common a decrease in GAGs formation. Our observations cannot identify the actual mechanism which gives rise to hydrocephalus, as our animals presented both a decreased concentration of GAGs and anomalies in the configuration of the posterior cranial fossa.

On the other hand, the two mechanisms proposed to explain the hydrocephalus are not necessarily mutually exclusive, and their combined influence cannot be ruled out.

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