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SEM patterns of spontaneous neural tube defects in chick embryos at the end of incubation

Canavese B, Colitti M and Oberosler R

Institute of Animal Production, Faculty of Agriculture, University of Udine, Italy.

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Abstract

The exposed nervous surfaces of 21 day old chick embryos with spontaneous neural tube defects (NTD) were studied with SEM. The suitability of the model species chosen and the usefulness of SEM for exploring the peculiar characteristics of the NTD are stressed. SEM profiles relating to the typology of the defect are discussed and various observation sites are compared with the structural characteristics of the neurulation and the ependymal surfaces in normal chicks. Some of the profiles are described as successive phases of the phenomenon and specific structures believed to be characteristic are identified.

Introduction

Neural tube defects (NTD) are congenital anomalies of the central nervous system resulting from the failure of the neural folds to fuse and the absence of skeletal and cutaneous components on the dorsal midline of the cerebro-spinal axis, so that nervous tissue is found exposed to the external environment. The various phases of neurulation - neural induction and evolution of the neural folds, secondary induction, cellular mechanisms and the molecular basis of neurulation - are involved in this process (Schoenwolf 1982, 1983; Campbell et al 1986; Saxen 1989; Copp et al 1990; Schoenwolf and Smith 1990).

The various opinions about the terminological and semantic approach to this wide and complex group of defects have been widely discussed (Duhamel 1966; Warkany 1971; Lemire et al 1978; Siebert et al 1990). In man, NTD are described as forms of "anencephaly" in which errors in the closure of the neural tube lead to the degeneration of the brain tissue, or as forms of spina bifida where the neural folds do not fuse in the spinal column. Craniorachischisis is skeletal fissure which extends from the

cranial region along the spinal column and is seen in forms of olocranic anencephaly. The open spina bifida in man includes forms differing in aspect and severity; closed spina bifida with neural lesions "hidden" by the integument layer is not recognized. Encephalocele is brain protrusion covered by the meninges and the skin is often considered to be a separate form. The term exencephaly is reserved for cases where the protruding brain tissue has not begun to degenerate and, in animals, generally indicates a situation in which the defective closure is associated with a juxtaposed and distorted growth of the neuroepithelium, as seen in every exencephalic embryo with an abnormally "exposed" brain.

It is known that the closure of the neural tube is complete after 27 days of gestation in man, 10.5 days in mice and 11.5 days in rats (Cockroft 1991); the chick embryo neural tube begins to close at stage 8 (26-29h), but the process is completed only about stage 12 (45-49h)[Bancroft and Bellairs 1975]; the closure of the anterior neuropore is considered complete by stage 12, while at this stage the fusion of the neural folds and the closure of the posterior neuropore is still underway (Portch and Barson 1974); secondary neurulation is a process involving the caudal regions of the vertebral axis and differs from neurulation in general because it does not involve the formation of neural folds but a cordon full of mesenchymal cells which become progressively canalised to form the origin of the neural tube (Schoenwolf 1984); the length of gestation of many animal species is considered too short to allow, in cases of anencephaly and open spina bifida, the successive degeneration of the abnormal neural tissue.

However, still it is not clear if the anomaly is due to the failure of the neural tube to close (primary error theory proposed by Von Recklinghausen 1886), or a secondary breaking (hydromyelic theory of Morgagni 1769) of a previously closed neural tube (Gardner 1961; Ganchrow and Ornoy 1979; Clark and Scothorne 1990). A recent study by Marin-Padilla (1991) introduced a new hypothesis to the origin of neural defects - that they are part of a heterogeneous group of cephalic axial skeletal-neural dystrophic disorders. It is proposed, as the common anomaly at the origin of the diverse malformations, an early paraxial mesodermal insufficiency. Recently, Padmanabhan (1984, 1990, 1991) has described exencephaly experimentally induced in rats after the neural tube had closed. The neural defect was accompanied by cranial and spinal skeletal dysmorphosis and the observation by LM and SEM revealed cellular and subcellular alterations of the neuroepithelium, the choroidal plexus and the mesenchyma in the defective area. Padmanabhan (1991) also demonstrated how, after appearing, the exencephaly evolved inexorably towards anencephaly and that the damage to the neural tube and the surrounding mesenchyma by a single dose of cyclophosphamide administered on the 12th day of gestation in rats caused irreversible deterioration of these tissues, even when an attempt was made to experimentally prolong pregnancy.

The NTD generally begin at the two extremities (anterior and posterior neuropores) of the dorsal midline, along which the neural folds fuse; this strengthens the belief that these defects are the result of abnormal closure. Campbell and Sohal (1990), in white peking duck, demonstrated that the NTD due to secondary opening by surgical micro-incision were less frequent in the cervicothoracic tract in comparison with the cephalic

and thoracolumbar tracts because the percentage of the defects healing in the former tract was higher. They specified that the usual and frequent location of human NTD at the rostral or caudal end of the primary neural tube could be due to other factors which were not limited to the position of the neuropores.

In order to further clarify the pathogenesis of NTD, it should be noted that several studies of embryos have shown that the neural and tegumentary epithelium in the defective area are closely associated as if normal fusion followed by separation had never occurred and that the surgical reopening of the neural tube of 3 day old chick embryos causes an NTD with new fusion of the neural and tegumentary epithelium within 24 h.

Earlier studies (Canavese et al 1979; Colitti and Canavese 1989) have shown that, particularly in the chicken, the spontaneous malformations can normally be observed when opening eggs unhatched at the end of a normal incubation cycle and the NTD found are an abundant source of material for study. The aim of the present work was to use the embryos with NTD obtained as described above as "model" samples and to apply exploratory scanning electron microscopy to observe the structural variations which are connected to the pathogenesis of the defect.

Material and Methods

Chicken eggs ("Marchall" and "Arbor" broilers) unhatched by the 21st day of incubation were quickly opened close to the incubators and 26 live embryos with spontaneous NTD and several controls were collected and examined.The malformed embryos were classified as merocranial or olocranial anencephalic according to Lemire et al (1978). The definition of exencephaly is reserved only for those forms which, in the absence of facial morphological alterations, present an apparently normal but exposed cerebral mass. Our study, with chicken embryos, never revealed forms of spina bifida, apart from the forms of olocranial anencephaly because in these the extension of the defect is also myelodysraphic. Before being sampled, the embryos were anaesthetised with ether; however, in many cases, natural death occurred a few minutes after the eggs had been opened. The samples of exposed brain and spinal cord collected for SEM observations were fixed in neutral formalin 10%, rinsed in phosphate buffer (pH 7.4), dehydrated in increasing concentrations of ethanol, dried in a critical-point drier with carbon dioxide and then sputter coated with gold (Sputter Coater S150B). The samples were observed under a Cambridge S250 Scanning Electron Microscope.

Results and Discussion

The unusual and original expression of the NTD causes the external exposure of the nervous tissue and the possibility of exploring the NTD surfaces with SEM. The NTD, i.e. the defects before closure, occur by day 3 of incubation, but from that date until hatching, when they can be observed, there remains enough time for some diversification in the profiles; the same can be said of the secondary NTD which, having different times of occurrence, have good reason for presenting structural differences.

The topographical element cannot be overlooked: differences are evident whenever SEM observations are made of adjacent areas of tegumentary, intermediate and neural epithelium. However, the profiles presented are not necessarily to be referred to an NTD typology; they can be common to several forms of NTD and be found contemporarily when exploring different areas of the same subject. However, the profiles have been interpreted according to the teratological severity because the various profiles can represent aspects of increasing severity in relation to successive pathogenic events. This compromise is compounded by the absence of an animal model which would allow the defect evolution to be investigated.

The removal of the cranial skull and part of the meningeal layers (Fig. 1) allows a differentiated observation of the osseo-meningeal and nervous surfaces in control subjects (Figs. 2 and 3). SEM clearly shows the frequent irregularity of the NTD surfaces. In fact, scanning electron microscopy stresses the images described with light microscopy. For example, in connection with the altered process of morphogenesis and the degeneration, the vasculo-connective phenomenon, which is quite often found associated with forms of anencephaly, produces ridges and mammillation, microcavities and grooves of various sizes (Figs. 4-5). The abnormal and confused vascular component of the defect often results in large, haemorrhagic sacs which become particularly wide. They tend to move towards the surface and empty their contents externally after the thin parietal tissue layers break. It is thus possible to directly observe by SEM the erythrocytes and leucocytes outside their normal environment, together with dead cells and fragmentary heterogeneous material (Fig. 6).

However, the early periods which precede the neural tube closure have been extensively investigated, as externalized surfaces, by the SEM. In addition, the description of the ependymal, ventricular and medullary walls in normal subjects with closed neural tubes obviously refers to internal surfaces, which, in the case of open NTD, become externalised. It is possible that, during the development of NTD embryos, many structural characteristics of the neural and ependymal surfaces can remain constant or develop so that they are found either altered and/or dislocated in various places. It should be noted that regional variations in the cellular surface have already been described during the differentiation of the epiblast of the chick blastoderm and these become clearer as development proceeds. In particular, the cells destined to invaginate have few microvilli but more numerous globular and vesicular projections when compared with cells destined to form the future ectoderm covering the embryo (Bancroft and Bellairs 1974). The external layer of the neural plate, future ependymal cells (Otani and Tanaka 1988), are flatted with small and irregular spherical protrusions along the cellular borders. In some cells it was possible to observe single cilia, recognizable by their uniform diameter (0.15-0.19μm) and the usual periciliary groove. Later on, the neural cells continue to have irregular and inflated surfaces with a large cilium located centrally; instead, the ectodermal cells, on the sides of the neural plate, accentuating the differences markedly, assume the form and the disposition of "flat plates" without evident cilia and large blebs (Portch and Barson 1974); overall, they are much wide cells, with prominent borders and surfaces rich with microvilli.

A fairly similar cellular aspect is also found in certain zones of the exposed neural surface in NTD embryos at the end of incubation. In fact, the neuroepithelial layer is

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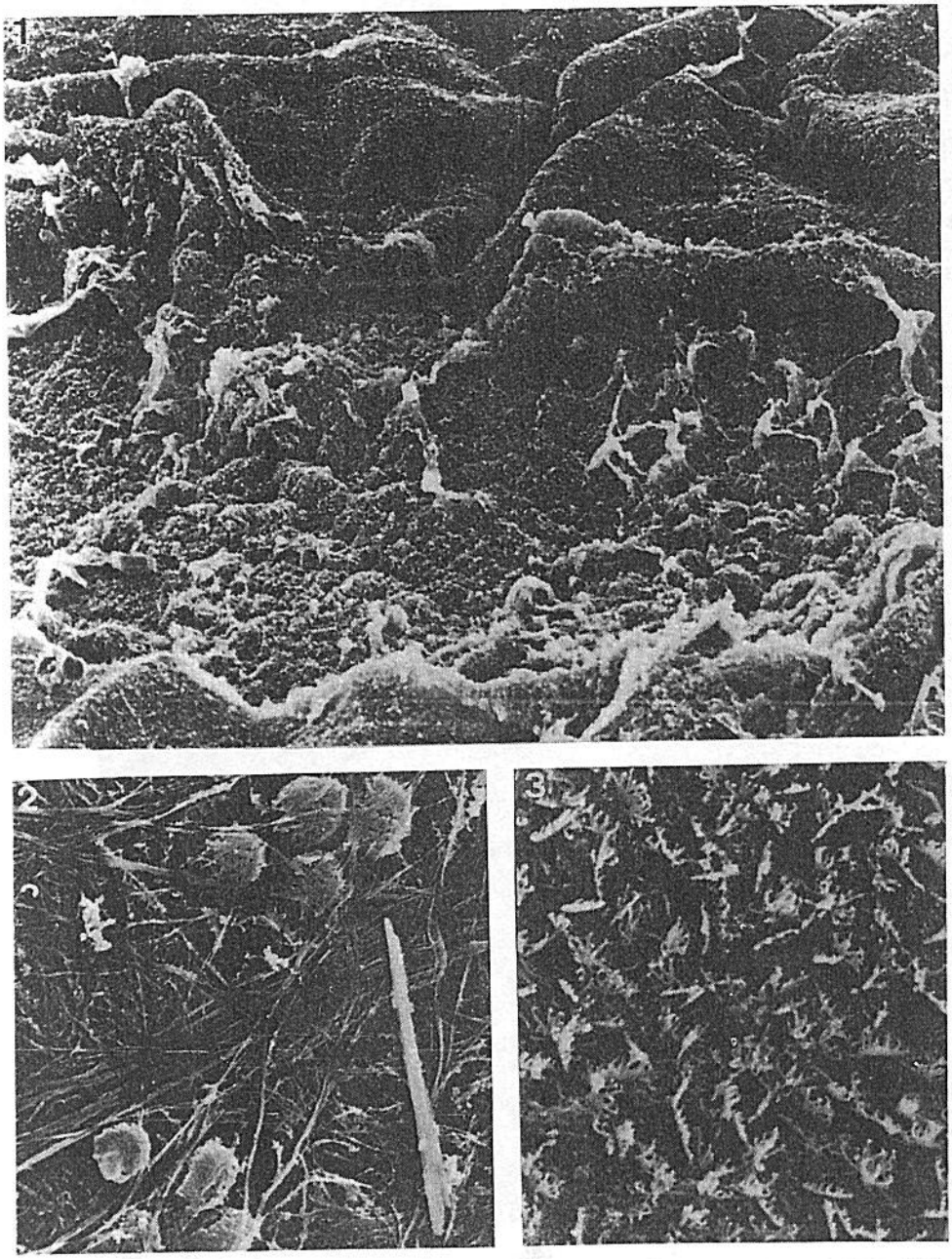
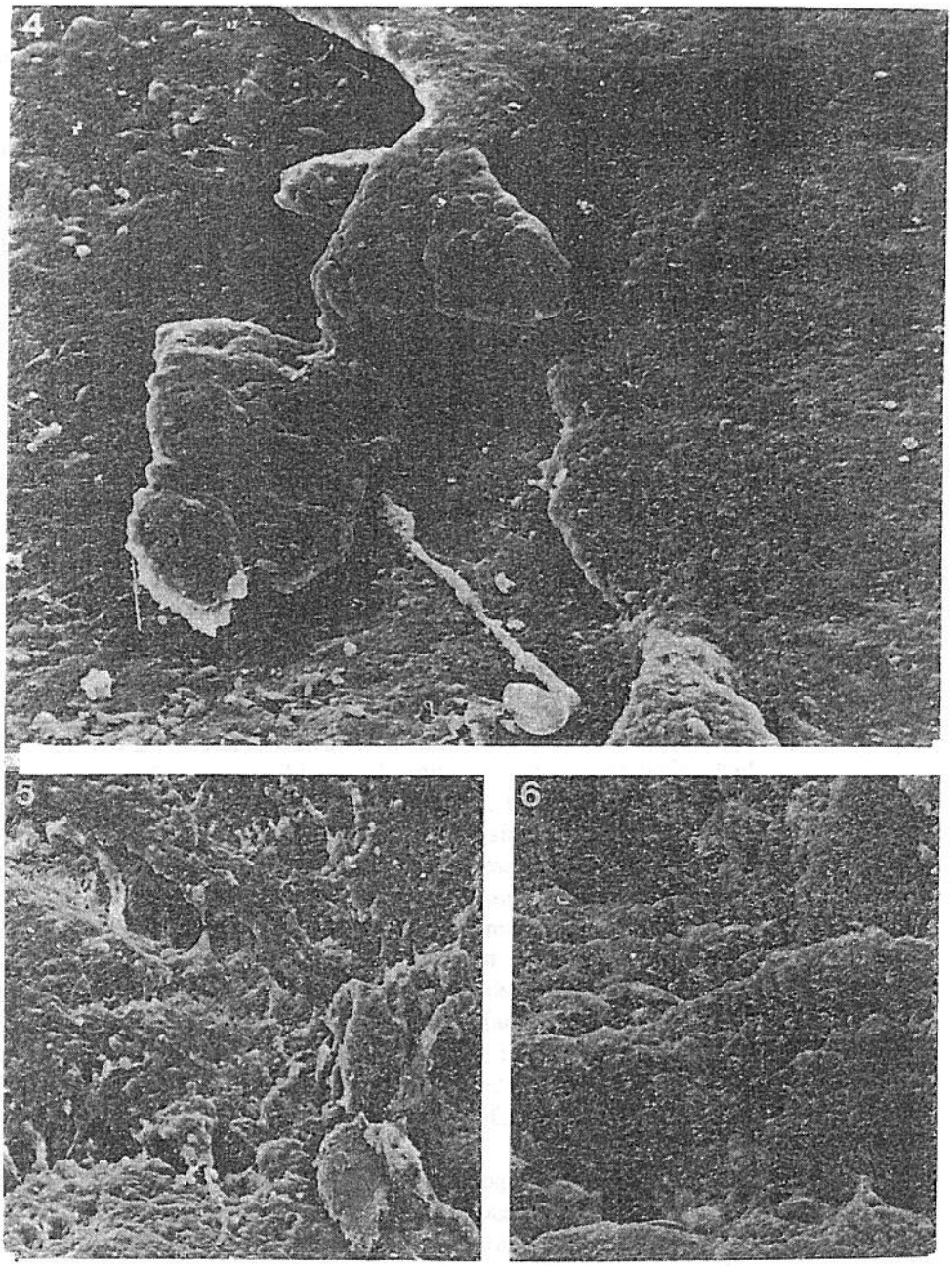


Figs. 1-3: Chick embryo after 21 days incubation. Control. 1. Meningeal and nervous surfaces. 260x. 2. Internal bone surfaces from the cranial vault. 1300x. 3. Detail of the meningeal surface. 5000x.



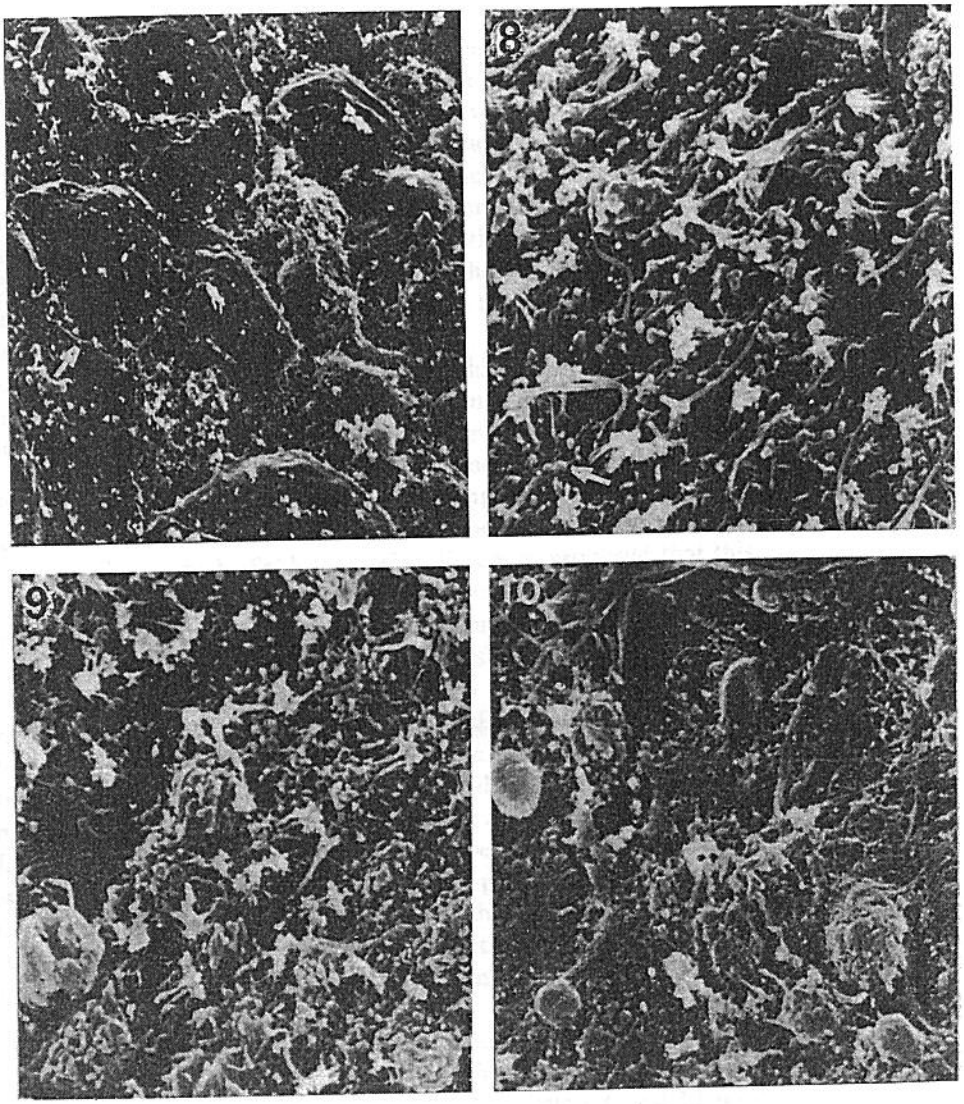
Figs. 4-6: Chick embryo after 21 days incubation. Merocranial anencephaly. 4. Overall view of surface irregularities. 320x. 5. Surface unevenness. 170x. 6. Mixed red blood cells and debris in a haemorrhaging cavity. 900x.

still composed of numerous cells which are more or less swollen, have microvilli on the surface and, in particular, in the intercellular spaces, as well as other cells undergoing decomposition and disaggregation material. The cellular borders are well defined, but the intercellular spaces increase particularly in the zones with dying cells. The neural nature of these cells and the presence of beaded (Schoenwolf 1983) or third type (Bancroft and Bellairs 1974) threads (Fig. 7, arrows) are noteworthy.

With the appearance and the progressive elevation of the neural folds, certain specializations of the cellular surfaces become more evident and significant (cilial projection, microvilli, blebs and surface folds). These beginnings underline that, once developed and distributed according to a specific topography, these aspects become characteristics of the ependymal and choroidal plexus surfaces. Since the introduction of SEM, the surface structures of mammalian and avian cerebral ventricles, including the choroid plexus, have been studied extensively in embryo-fetal and adult subjects (Chamberlain 1973; Mitchell 1980; Allen et al 1978 and 1981; Otani and Tanaka 1988). The overall data highlight how the microvilli, single cilia, tufts of cilia and other polypoid-like cellular projections, with their regular distribution and specific topography are inherent aspects of the surfaces under observation. Otani and Tanaka (1988), using SEM to study the development of the choroid plexus anlage and supraependymal structures in the fourth ventricular roof plate in human embryos, focused attention on the modifications of the surfaces and the relative regional variations, and in particular recognised two groups of morphologically different supraependymal cells (SE cells): type 1 SE cells with spindle or teardrop-like bodies; type 2 SE cells with globular and numerous fine pseudopodial processes.

Alongside zones with well defined cells which retained the above-mentioned characteristics, in our preparations it was possible to observe extensive areas which had pronounced modifications, although still maintaining the generic aspect of ependymal surface. As can be seen from figures 8, 9 and 10, the profiles showed varying filamentary and globular structures (i.e. short filopodial-like structures, cilia with stubby ends, tufts of cilia amassed at their free ends, globular projections and unevenly distributed piles of irregular globules which confer an overall aspect of "multiberry"). The figures describe more situations and document the fragmentation of the various specialised surface structures. Figure 10, in particular, highlights how this type of debris, almost pulverising itself, can become a discontinuous layer on the surface. From these profiles, we are inclined to believe that the origin of this debris is the intense and progressive fragmentation of the filamentary formations and various projection types. The "beaded" aspect, visible in some places along the axis of the surviving ciliary formations, is some evidence for this (Fig. 8, arrows).

Along the edges of the already closed neural folds, the progressive development of a narrow zone of cells which are flattened with membranous "ruffles" between the surface ectoderm cells and the rounded cell apices of the neural ectoderm can be seen (Waterman 1976). An unusual aspect of this is composed of cytoplasmic or filopodial threads, slender projections (4-4.5x0.1-0.4μm) which extend from one fold to another and which would play a role during the final stage of the fusion of the folds (Bancroft and Bellairs 1974; Nagele and Lee 1980; Schoenwolf 1983).



Figs. 7-10: Chik embryo after 21 days incubation. Merocranial anencephaly. 7. Neurular details of the cells, still well conserved. 2100x. 8-10. Olocranial anencephaly. Various aspects and sequence of debris formation. 8. 2900x. 9. and 10. 2300x.

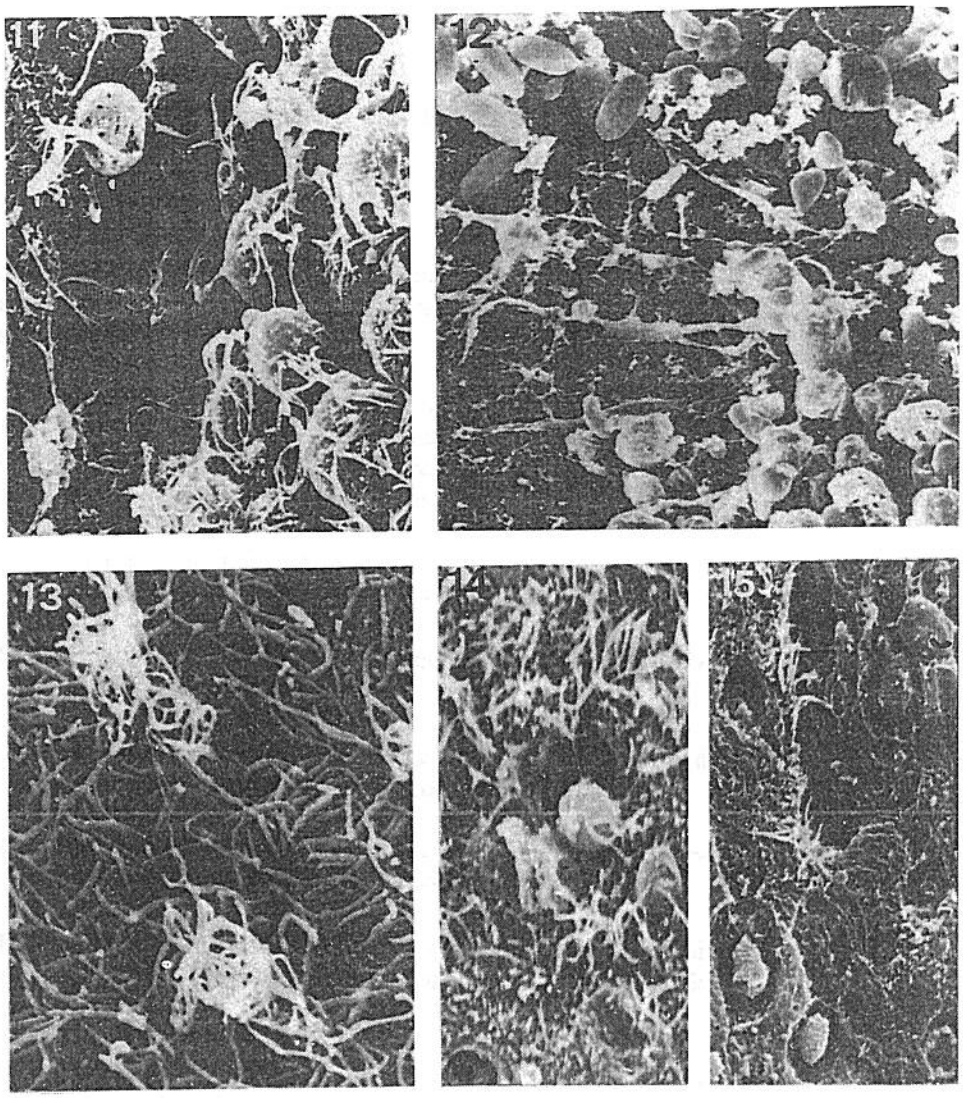
Aspects similar to those documented here, but of different nature, are also present in those parts of the preparation where tissue discontinuity occurs during the teratogenesis. In these spaces, structures similar to the threads can be seen. Instead, these are filamentary formations arranged so as to form a delicate web which extends from one side of the groove to the other, and also connecting with the remaining filamentary formations of the border cells. The cellular changes in the surrounding area are numerous and gradual. The following are examples of what can be observed : globular cells with smooth surfaces and few or no microvilli and cilia, others which are flattened with dense microvilli and increase intercellular spaces and others still, on the point of separating from the rest of the population or already free in the discontinuous space and in advanced state of degeneration (Fig. 11).

The debris is a characteristic of the neural tube lumen. This extracellular material should not be confused with the matrix; it is already present in the presumptive neural plate area, in the neural groove and can also be found in the neural tube lumen at any development stage. It appears to be composed partly of fragments of dead and degenerating cells expelled from the tissue, and partly of decomposing mid-bodies. The presence of large quantities of debris in the neural tube lumen was first recorded in rats by Freeman (1972) and in mice by Schluter (1973). Freeman proposed that this material was formed by pinching off the apices of the cells during the rolling up of the neural tube. Bancroft and Bellairs (1975) described two different types of debris; one deriving from the ejection of dead cells and the other consisting of discarded mid-bodies. It is well known that cellular death occurs continually in a normal embryo and this has an important morphogenic role, while the mid-bodies are remnants of the mitotic spindle which are eliminated at the end of telophase, and so it is possible to evaluate the mitotic activity in a region by examining under SEM their distribution on a tissue surface. In fact, it is easy to correlate the high mitotic activity between the cells which surround the neural tube lumen with the large number of mid-bodies present in the same region. It is also possible, when examining the preparations by SEM, to observe the differences between the two types of debris in that, while the mid-bodies tend to maintain a standard size (1/2-3/4 μm in diameter), the cellular debris can be composed of entire cells or fragments of varying dimensions (Bancroft and Bellairs 1974).

The debris observed on NTD surfaces have the characteristics of the above mentioned cellular debris (Figs. 11-12) and that which probably comes from the fragmentation of the various structural details shown in figures 8, 9 and 10. Figures 6 and 12 show how the various types of debris with RBC morphofunctionally retained can coexist.

Tufts of cilia are, as can be seen, typical components, but are more commonly observed on the ependymal surfaces and in some zones on the exposed NTD surfaces where they sometimes assume exuberant characteristics (Fig. 13). However, the cilia are also cellular surface details which, in the open NTD, can be liable to change (Figs. 14 and 15).

The NTD have been interpreted as defects caused by arrested development. In this regard, it should be specified that the failure to attain a given stage of morphogenesis is not accompanied by blocked histogenesis, as would be suggested by the differentiated neuroglial cells present in sections observed under LM. The variety in the



Figs. 11-15: Chick embryo after 21 days incubation. 11-12. Merocranial anencephaly. Discontinuity in the neural surface and cellular debris. 11. 2400x. 12. 900x. 13-15. Various ciliary aspects. 13. Olocranial anencephaly. 3000x. 14-15. Merocranial anencephaly. 14. 1400x; 15. 2000x.

structural profiles that SEM can demonstrate shows that the teratological process is very complex.

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Address for Correspondence:

Canavese Bartolomeo
Department of Animal Production Sciences
Via S. Mauro, 2
33010 Pagnacco (Udine)
Italy.