行政院國家科學委員會補助專題研究計畫成果報告

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※ B 型流行性感冒病毒感染造成胚胎畸形的機制探討 ※

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Influenza B virus-induced eye and brain malformations during early chick embryogenesis and localization of the viral RNA in specific areas

Short running title: Effect of influenza B virus infection on embryogenesis

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Abstract

Influenza is one of the most significant and prevalent viral infections worldwide. The potential effect of influenza on early embryonic development, however, is largely unknown. We report here the sequelae of influenza B virus infection on early chick embryogenesis. Chick embryos at Hamburger-Hamilton stage 9 were infected with influenza virus B/Taiwan/25/99 by injection *in ovo* under the blastoderm of 40 μL at 5 x 10^8 p.f.u./mL in EMEM. Gross malformations of the eye and the brain were observed after 48 hr, ranging from 40% to 64% among 14 infected batches. In the eye region, unilateral malformation, growth retardation, and absence of optic primordia were found. In the brain, anencephaly and repetitive foldings and segmentations were observed. Histological analyses showed extensive degeneration in the neuroectoderm and aggregates of cells indicating heterotopia in the mesenchyme. In some cases, unilateral thickening of the head surface epithelium was found. *In situ* hybridization with positive and negative RNA strands as probes for the HA segment of influenza B revealed that the viral RNAs were extensively located in the head surface ectoderm and in the lung bud. In the developing brain, the viral RNAs were specifically located in the rostral neural retina, habenular area, mid-thalamus, and rhombencephalic neuroepithelium. These data showed that

influenza B virus, given direct access to the developing embryos, could cause teratogenesis, presumably via the selective targeting of viral nucleic acids. Our data also suggest a concern on maternal influenza B virus infection during early pregnancy, if transplacental passage occurs.

Abbreviations:

anp, anterior neural plate; ANR, anterior neural retina; CNS, central nervous system; DI, diencephalon; HN, habenular nucleus; L, lens; LB, lung bud; NR, neural retina; OP, optic primordium; OV, optic vesicle; PROS, prosencephalon; RE, rhombencephalon; SC, spinal cord; SE, surface ectoderm; TH, thalamus.

Keywords: influenza B virus, embryogenesis, teratogenesis, in situ hybridization

Introduction

Influenza viruses remain one of the most significant and prevalent pathogens worldwide and many efforts have been made in describing the molecular aspects of the viruses, in elucidating the epidemiology and modes of spread, and in developing strategies for prevention and treatment. Nevertheless, whether maternal influenza virus infection will lead to teratogenesis in the fetus and how the virus will affect normal embryogenesis are largely unsettled.

Prenatal exposure to influenza epidemics has been reported by epidemiological studies to increase the risk of adult schizophrenia in Finland (Mednick et al, 1988), England and Wales (O'Callaghan et al, 1991), and in Japan (Kunugi et al, 1992). However, a study in the United States was negative (Torrey et al, 1988) and a Scottish study showed no difference (Kendell and Kemp, 1989). The contradictions were likely resulted from methodological defects in gathering information about maternal infection based on maternal memory or the quality of later psychiatric diagnosis (Wright et al, 1993). Numerous efforts also have been made to elucidate acute encephalopathy and postinfluenza encephalitis, based on serological detection of maternal antibody or, more directly, the spread of viral antigens in the maternal serum

(Reviewed by Schlesinger et al, 1998). To our knowledge, none of the previous studies located directly the nucleic acid of influenza virus in the fetus. Thus, the effect of direct access, replication, and targeting of influenza virus genome in the developing CNS has not been shown. Previous studies also concentrated on maternal exposure during the second trimester (Barr et al, 1990; Sham et al, 1992), which could have lost the potential effect on early organogenesis in the fetus. Furthermore, excessive drug consumption contingent on infection may have complicated the acceptance of a viral disease as teratogenic in an epidemiological study (Karkinen-Jääskeläinen and Saxén, 1974).

With the difficulties in studying human objects, attempts to correlate influenza virus infection with congenital neuropsychiatric diseases have to be shifted to the animal models. Studies on animal models, however, have not concentrated on the effect of influenza infection to embryos. Davis and Kornfeld (1986) tried to establish an influenza B virus-infected mouse model of Reye's syndrome by intravenous inoculation into 3-week-old Balb/c mice. The inoculated mice exhibited some symptoms as seen in the human Reye's syndrome. In an earlier study, with the same infection procedures, the influenza viral hemagglutinin, neuraminidase, and nucleoprotein antigens were detected in hepatocytes and brain capillary cells (Davis et

al, 1983). Nevertheless, they found no virion in the mice as detected by electron microscopy (Davis and Kornfeld, 1986). The authors suggested the possibility of defective or abortive viral infection, but no further evidence was provided. In another report by Mori et al (1999), a neurotropic strain of influenza A virus was administered through stereotactic olfactory bulb injections into 4-week-old mice. The injection resulted in selective immunohistochemical localization of influenza A virus antigens in the brain, which was further confirmed by RT-PCR analyses. An *in situ* localization of the viral genome and its replication products remained not provided. To elucidate influenza virus-induced neuropathogenesis, a direct localization of the distribution and replication of viral genome will be more convincing than antigen or antibody detection. The effect of influenza B virus on chick embryonic development and the distribution of viral genome in the developing brain, to our knowledge, have never been reported.

Influenza B viruses are mainly human pathogens (Murphy and Webster, 1990) and, like influenza A viruses, cause periodic epidemics and pandemics worldwide. In the present study, we investigated the effect of influenza B virus on early embryogenesis using chick embryos as a model, and directly localized by *in situ* hybridization the presence and replication of viral RNA in selective areas of the developing brain.

Results

Gross malformations and histological observations in the eye and the brain

Following influenza B virus infection, gross malformations were found exclusively in the eye and the brain (Fig. 1). Gross malformations in the eye were found in 69 (52%) out of a total of 133 infected embryos from 14 batches of experiments (Table 1). In the brain, there were 68 (51%) cases of malformations. There were 34 (26%) infected embryos in which malformations were found in both the eye and the brain. Regardless of the location, malformation rates ranged between 40% to 64%, in contrast to the overall 3% found in the brain of the control embryos where mock infections were conducted without influenza B virions. Death rate was 32% in the experimental embryos, in contrast to the 8% in the controls. Gross malformations in the eye included unilateral growth retardation, distortion or diminution of the pigmented retinal epithelium, and unilateral absence of the optic primordium. In the brain, anencephaly and repetitive folding (not shown) of the neuroepithelium were found, suggesting neural tube closure defects and abnormal segmentations. No apparent abnormality in gross morphogenesis was found in the other regions of the infected embryos.

Histological analysis revealed twisting of the neuroepithelium in most of the embryos following influenza B virus infection (Fig. 2). Signs of extensive cell death were found in most of the influenza B virus-infected chick embryos in the head region. Heterotopia in the head mesenchyme was common and, in some cases, unilateral thickening of the head surface epithelium was observed.

Selective localization of influenza B virus RNA

To elucidate the mechanisms underlying influenza B virus-induced abnormalities, we performed *in situ* hybridization at 48 hr after infection to locate directly, as indicated by the presence of HA segment, the distribution of the viral RNA. Influenza B virus RNA was found extensively in the surface ectoderm and in the developing lung bud (Fig. 3). In the brain, the viral RNA was selectively located in the habenular area of the forebrain, the mid-thalamus, the hindbrain, and in the anterior part of the neural retina. Signals of symmetrical distribution of the viral RNA were found in many of the infected embryos. We used both positive and negative strands of RNA probe to localize not only the presence, but also the replication of the viral RNA. The distribution of positive strands was comparative to that of the negative strands,

indicating no difference between the areas of infection and the areas of replication in the developing embryos.

Restriction of viral RNA distribution following embryonic development

To follow the dynamic distribution of influenza viral RNA during embryonic development, we investigated the infected embryos at 12, 24, 36, and 48 hr following infection (Fig. 4). At 12 hr after infection, the viral RNA was located in the surface ectoderm as well as in a broad area of the anterior neural plate. Distribution of the viral RNA was restricted in selective areas of the brain by 24 hr following infection. The restriction appeared to continue in the embryos at 36 and 48 hr after infection. By 48 hr following infection, as the embryos developed, the brain and the optic primordium increased in size and the areas where the viral RNA was located appeared to be further restricted.

Discussion

In the present work, we demonstrated that, given direct access and sufficient amount of influenza B virions, malformations in the eye and the brain could be induced in a substantial portion of the chick embryos infected at Hamburger-Hamilton stage 9 (Hamburger and Hamilton, 1951) during early embryogenesis. The observed malformations could be resulted from lack or insufficiency of immune protection, for that the blood-islands are present only in the posterior half of blastoderm from stage 8 (Hamburger and Hamilton, 1951). The embryonic immune-competent cells might probably not be totally functional at stage 9, especially in the anterior part of the embryo where the malformations were exclusively found.

The malformations found in the present study suggest a concern on maternal influenza B virus infection during early pregnancy, despite that a direct access of influenza viruses to the early embryos is still to be confirmed in the eutherians. Maternal immune protection in the eutherians is generally active and some structural features of the placenta can be potential impediments to transmission; both were not present in the chicken model used in this study. However, there have been reports indicating transplacental influenza A infection as evidenced by cord blood HA1 antibody titers

and lymphocyte proliferative responses (Ruben and Thompson, 1981), and by cord blood hemagglutination inhibition and complement fixation antibody titers (McGregor et al, 1984). Physical defects are known to occur in the trophoblast layers at all stages in gestation (Burton and Watson, 1997). Early maternal recognition of influenza infection may fail, especially in the immune-compromised mothers. Therefore, we believe that potential transplacental transmission of influenza B viruses can not be excluded. The malformations observed in the chick model may still apply to the eutherians, including the human.

The extensive localization of influenza B viral RNA in the head surface ectoderm and the lung bud suggests that infection in eutherian embryos will be through amniotic fluid and not necessary via the connecting stalk. Apparently, further studies by direct *in situ* localization of viral genome and its replication products in the placenta, amniotic fluid, cord blood, and the embryos are needed to settle this issue.

The localization of viral RNA and its replication intermediates in the habenular area of forebrain, mid-thalamus, hindbrain, and in the anterior part of neural retina correlates with a selective involvement of regions in the brain implicated in neuropsychiatric disturbances. Our data also suggested no defective or abortive viral

infection, at least in certain selective areas of the developing brain. Furthermore, results by wholemount *in situ* hybridization at 12, 24, 36, and 48 hr after infection revealed developmentally restricted distribution of viral RNA in the neural ectoderm, without interference with viral propagation. Such developmental restriction is likely due to the gradual gaining of immunocompetence as the embryo develops, which leaves selective virus dissemination depending on anatomical sites (Stevensen et al, 1997a, b). Alternatively, only the cells in selective areas of the developing brain maintain receptor binding specificities toward sialo-sugar chain linkage (Xu et al, 1996) and the viral infection in the other areas becomes abortive.

Influenza virus-induced cellular pathogenesis have been shown to involve in the production of cytokines including IFN-α, IFN-γ, TNF-α, IL-6, GM-CSF, IL-1α, IL1-β, and IL-12 (Hennet et al, 1992; Monteiro et al, 1998) and in the activation of latent TGF-β (Schultz-Cherry and Hinshaw, 1996). Together, these factors contribute to the neurovirulence and eventually lead to the destruction of cells through necrosis (Campen et al, 1989) and apoptosis (Hinshaw et al, 1994; reviewed by Nagata, 1997). Interestingly, TGF-β has been shown to regulate a broad range of cellular functions during embryonic development, including proliferation, extracellular matrix secretion and adhesion, terminal differentiation and specification

of developmental fate (reviewed by Moses and Serra, 1996; Whitman, 1998). TGF-β also involves in apoptotic patterning of the neural crest cell during rhombomere segmentation (Graham and Lumsden, 1996). Therefore, we assume that the unilateral thickening of surface ectoderm, repetitive segmentation of the brain, and heterotopia in the mesenchyme found in the present study are likely to be induced by the TGF-β signal transduction pathways. These possibilities, however, remain to be confirmed.

Materials and methods

Virus propagation and titration

Influenza virus B/Taiwan/25/99 was isolated from a local child patient who exhibited neurological symptoms during an epidemic in Taiwan. The virus was grown in the Madin-Darby canine kidney (MDCK) cells in Eagle's Modified Essential Medium (EMEM) supplemented with 10% fetal bovine serum, 2mM glutamine, 100U/ml penicillin, and 0.1 mg/ml streptomycin, at 37℃ in 5% CO_2. The virus titer was determined by plaque assay on MDCK cells. After titration, the influenza B virus was diluted in EMEM or DMEM to a concentration of 5 x 10^8 p.f.u./ml and stored in a - 70° C freezer until use.

Preparation and infection of chick embryos

Fertilized, pathogen-free chicken eggs were purchased from a local farm and incubated in 38.5°C for the embryos to develop until Hamburger-Hamilton stage 9 (Hamburger and Hamilton, 1951). To infect a chick embryo, an aliquot of 20 μl of influenza B virus in EMEM at 5 x 10^8 p.f.u./ml was carefully injected *in ovo* into the

space between the embryo and its underlying yolk. The needles for injection were gauge 30. Efforts were taken in avoidance of damage to the embryos. The virus-infected chick embryos were put back in 38.5℃ for further incubation until fixation. Chick eggs with mock-infection were used as controls and incubated in another oven to avoid contamination.

cDNA cloning of HA segment

To directly localize the presence and replication of influenza B viral RNA in the infected embryos, we cloned cDNA of the HA segment for negative and positive RNA probe synthesis. Total RNA was extracted by the TRIZOL Reagent (GIBCO-BRL) and reverse transcribed in a total of 20 μl of reaction mixture containing 5 μl of total RNA, 0.2 μM of random hexamer, 200 μM of the four dNTPs, 1x buffer and 10 U of AMV reverse transcriptase (Promega). The mixture was incubated at 42℃ for 1 hour, inactivated by heating at 72℃ for 20 min, and quickly chilled on ice. The prepared cDNA was amplified in a PCR thermocycler (Perkin-Elmer 2400), with each 50 μl reaction mixture containing 10 μl of the HA cDNA template, 20 pmoles of each primer, 0.25 mM of dNTPs, 5% DMSO, 1.5 mEq of Mg^{++}, and 2.5 units of Taq polymerase (Promega). The following cycling

parameters were used: 94℃ for 1 min, 55℃ for 1 min, and 72℃ for 1 min for 35 rounds followed by a final extension step of 72℃ for 7 min. The primers used to amplify HA segment were:

SZB^{+}: 5'-AGCAGAAGC-3'

BHA^{+}: 5'-CGAATCTGCACTGGGATAACATC-3'

BHA^{-}: 5'-TGCACCATGTAATCAACAACAA-3'

The amplified PCR product was then cloned into the pCR®II-TOPO vector (Strategene) following manufacturer's instructions.

RNA probe synthesis and in situ hybridization

In situ hybridization for tissue sections and wholemount embryos were performed according to previously published procedures (Cox et al, 2000). Both DIG-labeled positive and negative RNA strands were used to localize the presence of viral nucleic acids, as well as viral replication. The RNA probes were synthesized according to manufacturer's instructions (Boehringer-Mannhein). Briefly, the pCR®II-TOPO vector containing the HA insert were linearized with BamH1 and EcoRI for positive

and negative probe preparation, respectively. The linearized plasmid was then subject to *in vitro* transcription using either T7 or SP6 RNA polymerase to incorporate DIG-dUTP nucleotides. Before hybridization on experimental materials, the synthesized probes were used to probe nitrocellulose-bound influenza A and B virions extracted from MDCK cells. Both positive and negative probes hybridized with influenza B virions without cross-reaction with influenza A. The infected embryos were fixed with 4% paraformaldehyde in PBS, paraplast-embedded, and sectioned at 5 μm in thickness. The tissue sections were probed with synthesized DIG-labeled probes, washed, processed for binding of the alkaline phosphatase-conjugated anti-DIG, and color-detected with BCIP/NBT. A 1% safranine solution was used for the red background counterstain. For every run of *in situ* hybridization, the controls were treated under the same procedures to compare with the experimentals, only without virus infection. All positive results were performed at least twice for confirmation. To detect the dynamic alterations of influenza B virus-targeted areas *in situ* hybridization was also performed on wholemount embryos at 12, 24, 36, and 48 hr following infection.

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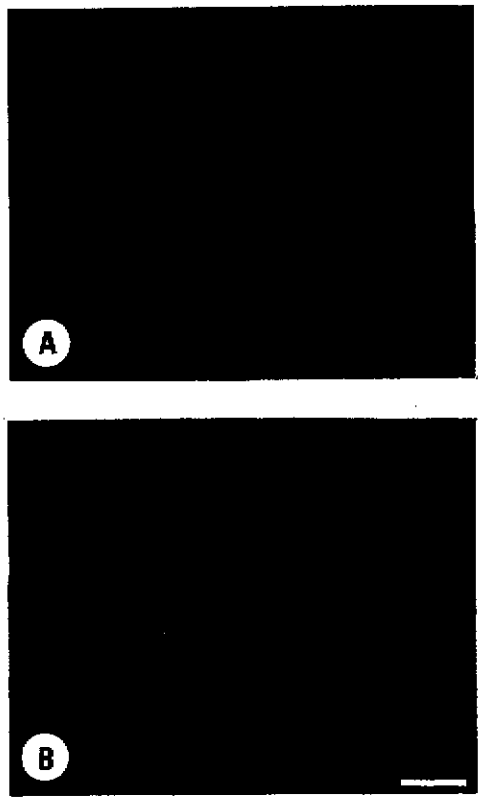
Figure 1 Gross malformations following influenza B virus infection. A, anencephaly indicated by arrows. B, unilateral malformation in the eye as indicated by arrowheads. The lower optic primordium did not develop a lens. Scale bars: 500 μm.

Figure 2 Tissue sections stained with Haematoxylin-Eosin from influenza B virus-infected chick embryos at 48 hr following infection. A, unilateral thickening of surface ectoderm and extensive cell death in the neuroepithelium. B, unilateral duplication of optic vesicle in a developing embryo. Abbreviations: SE, surface ectoderm; OV, optic vesicle. Scale bars: 500 μm.

Figure 3 Localization of HA segment of influenza B viral RNA at 48 hr after infection by *in situ* hybridization. A, extensive distribution of viral RNA along the surface ectoderm (arrowhead-indicated). B, localization of viral RNA in the lung bud. C, sites of viral RNA distribution in the rhombencephalon (indicated by arrows). D, areas of viral RNA distribution in the forebrain, including habenular nucleus, anterior neural retina and thalamus. Abbreviations: ANR, anterior neural retina; HN, habenular nucleus; L, lens; LB, lung bud; NR, neural retina; RE, rhombencephalon; SC, spinal cord; SE, surface ectoderm; TH, thalamus. Scale bars: 500 μm.

Figure 4 Developmentally restricted localization of influenza B viral RNA in 4 wholemount chick embryos at 12, 24, 36, and 48 hr respectively following infection. A, influenza viral RNA in the surface ectoderm and anterior neural plate found at 12 hr. B, distribution of the viral RNA at specific sites in the prosencephalon and diencephalon at 24 hr. C, viral RNA detected in only one side of the optic primordia at 36 hr. D, localization in specific sites within the eye and the rhombencephalon (indicated by white arrows) at 48 hr. Abbreviations: anp, anterior neural plate; DI, diencephalon; OP, optic primordium; PROS, prosencephalon; RE, rhombencephalon; SC, spinal cord; SE, surface ectoderm. Scale bars: A, 250 μm; B, 500 μm; C - D, 1 mm.

Bach no		No of infected embryos	No of observation	Eye malformation no (%)	Brain malformation no (%)	Both Eye & Brain malformation	Dead at fixation	normal
1	exp	12	12	5 (42%)	7 (58%)	5	3	5
	control	0	6	0	0	0	0	6
2	exp	12	7	4 (57%)	3 (42%)	3	2	3
	control	0	4	0	1	0	1	3
3	exp	12	9	5 (56%)	4 (44%)	3	2	3
	control	0	5	0	0	0	0	5
4	exp	12	10	6 (60%)	5 (50%)	3	3	2
	control	0	6	0	0	0	0	6
5	exp	12	11	5 (45%)	7 (64%)	3	4	2
	control	0	6	0	0	0	0	6
6	exp	12	12	7 (58%)	6 (50%)	3	5	2
	control	0	6	0	0	0	0	6
7	exp	12	9	4 (44%)	4 (44%)	1	2	2
	control	0	4	0	0	0	1	3
8	exp	12	11	7 (64%)	5 (45%)	3	4	2
	control	0	5	0	0	0	1	5
9	exp	12	7	4 (57%)	4 (57%)	2	3	1
	control	0	3	0	1	0	0	2
10	exp	12	9	5 (56%)	5 (56%)	1	2	0
	control	0	5	0	0	0	0	5
11	exp	12	10	6 (60%)	4 (40%)	2	5	2
	control	0	6	0	0	0	0	5
12	exp	12	9	4 (44%)	5 (56%)	1	4	1
	control	0	6	0	0	0	1	6
13	exp	12	10	4 (40%)	5 (50%)	3	1	4
	control	0	5	0	0	0	0	5
14	exp	12	7	3 (42%)	4 (57%)	3	3	1
	control	0	4	0	0	0	2	4
Total	exp	168	133	69 (52%)	68 (51%)	34 (26%)	43 (32%)	30 (23%)
	control	0	71	0 (0%)	2 (3%)	0 (0%)	6 (8%)	67 (94%)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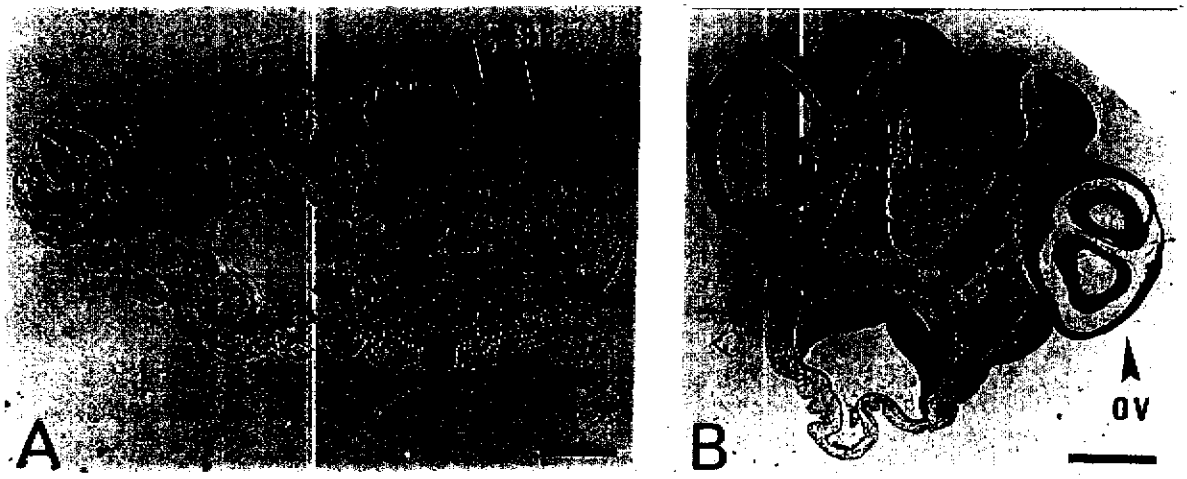
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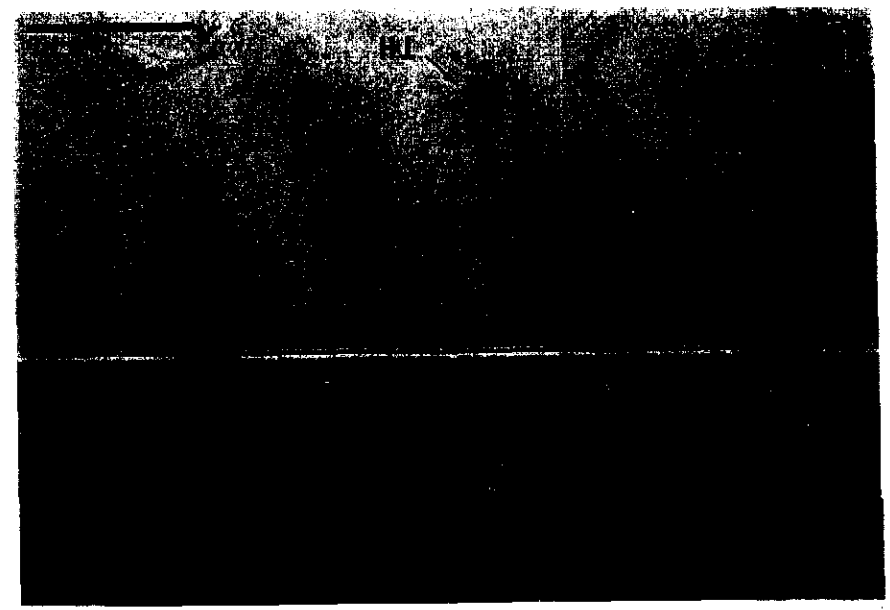

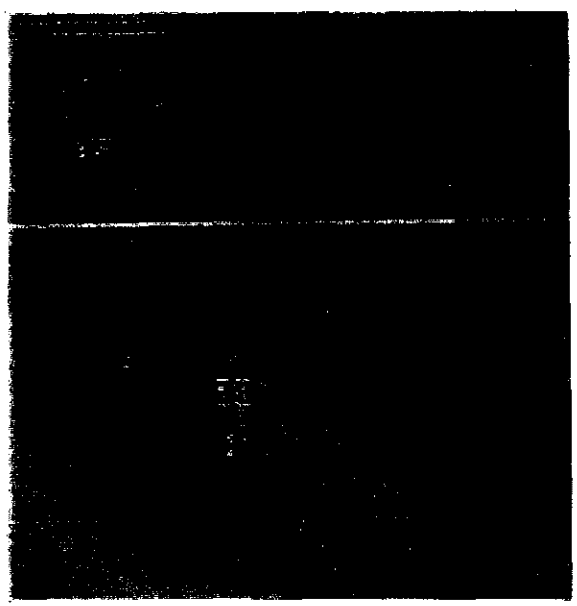

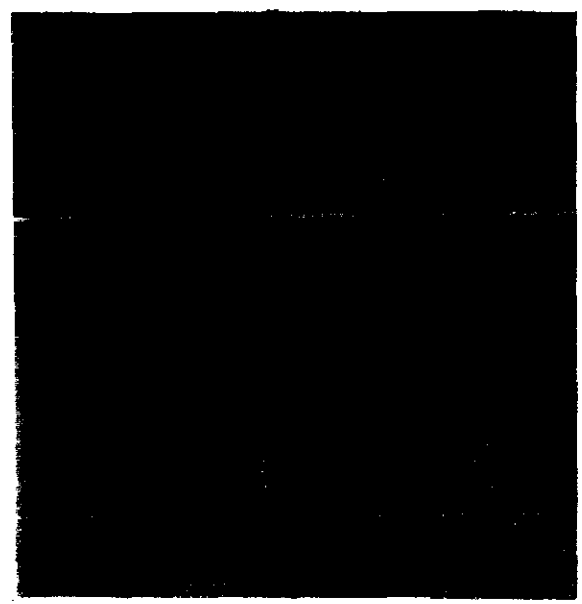

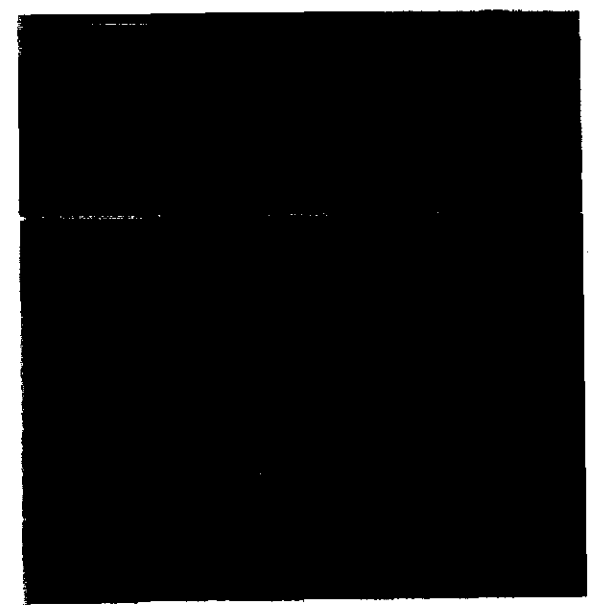

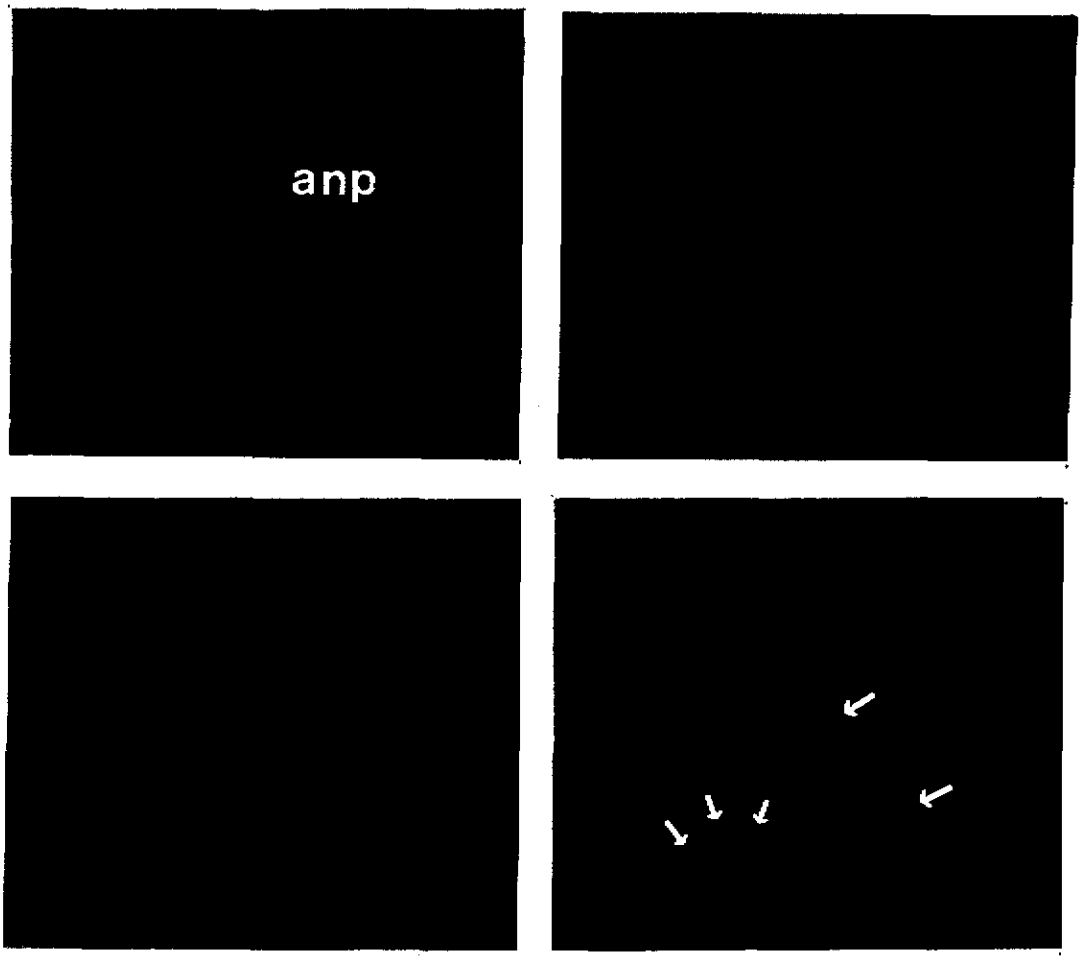
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