CYTOPATHOGENICITY OF INFLUENZA VIRUS (H_4 N_3)

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SUMMARY

Monolayer tissue cultures of chicken embryo fibroblast (CEF) cells infected with avian influenza virus isolate were examined by the hematoxylie and eosin (H & E) staining and indirect immunoperoxidase test for studying the cytopathogenic effect of the virus. Cytopathological changes which occurred in the uncleus of infected cells included nuclear and nucleolar hypertropy, chromatin margination and intranuclear inclusions. The most striking cytoplasmic change were the presence of perinuclear. eosinophilic inclusions at 22-36 hours post inoculation (p. i.) . Vacuolization, and granulation were also observed. Indirect immunoperoxidase (IIP) test demonstrated the localization of influenza virus antigens in infected cells. A positive peroxidase reaction observed in the nucleus and cytoplasm were similar to those shown in hematoxyline and eosin staining.

INTRODUCTION

Strains of influenza A viruses are capable of propagating in several types of primary cell cultures and established cell lines leading to induction of cytopathic effect (CPE) (1,2,3,4). Studies carried out on the formation of CPE reveal the presence of a relationship between the degree of CPE and the virulence of the infecting influenza virus strain.Highly pathogenic viruses produce CPE, both with and without trypsin, while viruses of low

virulence produce CPE only in the presence of trypsin (5, 6) The degree of CPE has also been associated with the infected cells (7). The paper reports the cytopathogenicity studies of the avian influenza virus H_4N_3 subtypes, which isolated from passerine birds .

MATERIALS AND METHODS

Virus:

Influenza virus A/magpie robin/ 23 / 89 isolated from passerine birds, were identified as H_4N_3 (8, 9) Stock preparations were made by inoculating a 10^{-3} dilution of concentrated virus suspension, into the allantoic sac of 10 – 11 day old chick embryos. After 48 hours of incubation at 37 C° the allantoic fluid were pooled and tested for hemagglutinating activity and bacterial sterility . The allantoic fluid containing virus had an infectivity of $10^{5.5}$ fifty percent tissue culture infective dose ($TCID_{50}$) per ml. The virus was stored at –70C° until it was used .

Cell culture and infection :

Monolayer of CEF cultures were grown on coverslips placed in tissue culture leighton tubes containing minimum essential medium (MEM) with 10% inactivated faetal bovine serum (FBS). CEF cells were inoculated with 0.1 ml of undiluted infectious allantoic fluid which contained approximately $10^{5.5}$ $TCID_{50}$ / ml. Tubes were incubated at 37 C° with MEM containing 5 ug per ml trypsin

Antisera

Hyperimmune serum was prepared according to the method of Alexander et al. (6)

Cytopathogenic Studies:

At timed intervals (0,2,4,6,9,12,16,19, 22, 24,36, 48, 72 & 69 hours) post inoculation, control and infected monolayers on coverslips were removed for studies of CPE. H.and E stain and IIP were used according

to the methods of (10 and 11).

RESULTS

Hematoxyline and Eosin Staining

Cytopathic effect was first observed at six hours p. i. with influenza A (H_4 N_3). The changes were observed in the nuclei of infected cells. The affected nuclei were purplish in colour, and mottled due to aggregation of chromatin . The first signs of granulation a ppeared at nine hours p. i., as diffused, fine uniform granules (Fig. 1).

At 12 hours p. i., small , poorly stained, single basophilic nuclear inclusions surrounded by a clear zone (Fig. 1). Nuclear changes seen at 24 hours included nuclear and nucleolar dissolution, nucleolar pyknosis and rupture of the cells. With further incubation, the number of cells with nuclear changes decreased. At 22 hours p. i., small, irregular and lightly stained eosinophilic cytoplasmic inclusions were observed in the perinuclear portion of cytoplasm (Fig .2) and they were also observed at 24 and 36 hours p. i. Numerous small vacuoles were observed at 48 hours p. i. Presenting a foamy appearance (Fig. 3) The morphological changes proceeded to complete destruction of cell layer about 72 to 96 hours p. i.

Indirect Immunoperoxidase

At two hours p. i. CEF cells fail to react positively either in intracellular spaces or on the surface the cell membrane. At four hours p. i., the cytoplasm of infected cells showed positive peroxidase reaction of infcted cells showed positive peoxidase reaction (brown colouration) whereas the nucleus appeared unstained . A marked peroxidase reaction localized in the nuclei, was first detected six hours p. i.

At nine hours p. i., localized areas of nuclei of CEF were stained brown. Fine aggregates of granular

character were found near the nucleoli . Enlarged nuclei were clearly visible with hypertrophied nucleoli, bordered with a brown demarkation line.

At 12 hours p. i., dark brown stained inclusions were clearly visible in the nuclei of infected cell cultures (Fig. 4). At 16 to 18 hours p. i., positive peroxidase reaction was revealed on nuclear membrane, as dark brown perinuclear, cytoplasmic inclusions in infected cells. An obvious increase in darkness of brown staining of perinuclear cytoplasmic inclusions was observed at 22-24 hours p. i. (Fig 5).

At later stages of infection, sever degeneration of cells set in vacuolization was observed and cells demonstrated intracytoplasmic inclusions at 48 hours p. i. The border of vacuoles showed dark brown staining. At 72 hours, a large proportion of the cells was sloughed off from the coverslips and weak positive peroxidase reaction was seen only in a few remaining cells.

DISCUSSION

An important characteristic of influenza virus as mentioned by (12) and (6) is that virulent influenza virus produce CPE in vitro with or without trypsin, while viruses of low virulence produce CPE only in the presence of trypsin . Trypsin was used in this study to induce cleavage of the hemagglutinin of the virus that had been previously characterized as low pathogenic virus (8 and 9).

In the present study the first changes in infected cells were seen in the nuclei, at six hours p. i., Affected nuclei showed clumping of nuclear chromatin At the same time, the IP revealed localization of virus antigen in the nuclei. Later the chromatin showed margination giving rise to thickened nuclear membrane. Nuclear changes caused by the virus were similar to those produced by some influenza A strain in calf kidney cell culture (1). Intranucler inclusions that were detected by H and E and IIP in the infected cells at 12 hours p. i. may have been formed by

condensation of basophilic material including nuclear chromatin into a single mass. These findings was simillar to results of (13) who detected intranuclear inclusions in cells infected with AIV strain A/FPV/Weybridge. The appearance of viral antigen initially

in the nucleus and later in the cytoplasm of influenza virus infected cells was confirmed by (14) by using fluorescent antibody technique. Cytoplasmic inclusions constituted the significant CPE observed in infected cells, and these consisted of a dense aggregated of granular cytoplasmic material. They may consist of viral protein material or mature virus particles. The persence of cytoplasmic inclusion was supported by IIP observations. These inclusions probably represented cellular proteins which had been altered as a results of viral action.This findings were similar to those produced by other influenza subtypes (3) . The vacuolization showed in infected cells was probably represents a mechanism of virus release. Rounding of the cells, granularity and vacuolization of cytoplasm were similar to those seen in research of (15). The early detection of positive peroxidase reaction in cytoplasm (at four hours p. i.) and in nucleus (at six hours p. I) of infected CEF cells corresponded to the observation of (14, 16).

Intracytoplasmic inclusions, which showed positive peroxidase reaction, were first detected as early as 16 hours p. i. This finding was not in accordance with the results obtained by (17). The results of the IIP test showed that the antisera prepared against virus isolates contained specific antibodies against the homologus antigen. This study provides information on the usefulness of the H and E staining technique in showing the morphological changes that occur in the nucleus and cytoplasm as a result of virus infection , it fails to show virus antigen localization. However, the IIP,which is a more sensitive serological test, confirms the H and E findings which simultaneously reveals localization of virus antigen that cause the stained changes in infected cells.

Fig. 1: Cytoplasmic granulation, Intranuclear inclusions Thick Arrow) and nuclear hypertrophy (Thin Arrow). 12 hours p. i. H and E stain. (X400).

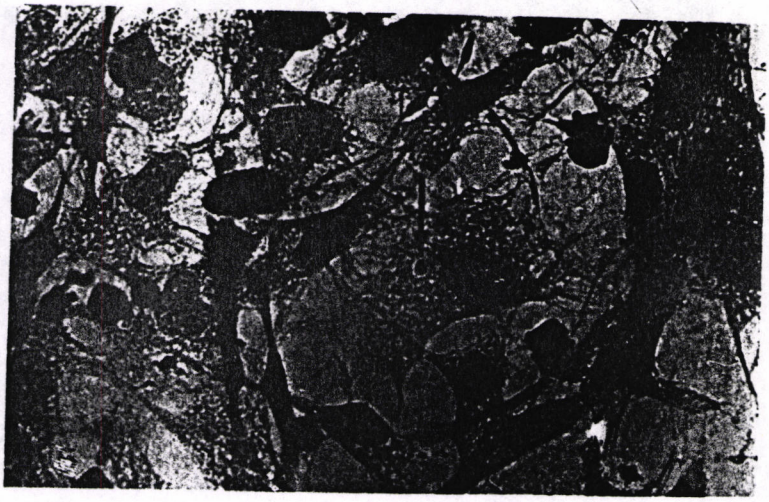

Fig. 2: Cytoplasmic inclusion (Thick Arrow), nuclear hypertrophy and pyknotic cells observed at 24 hours p. i. H and E stain. (x 400).

Fig. 3: Numerous vacuoles with foamy appearance and perinuclear inclusions observed at 48 hours p. i. H and E stain (x 400).

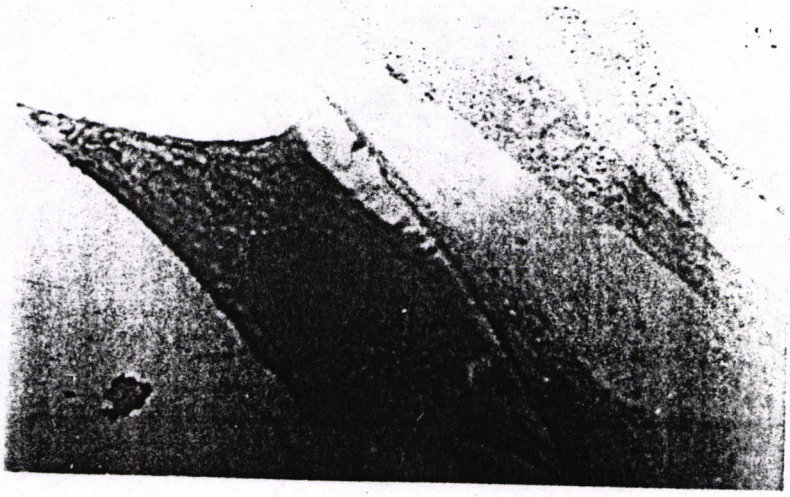

Fig. 4: Dark brown Immunoperoxidase stained intranuclear inclusions (Arrows), at 12 hours p. I (x 1000).

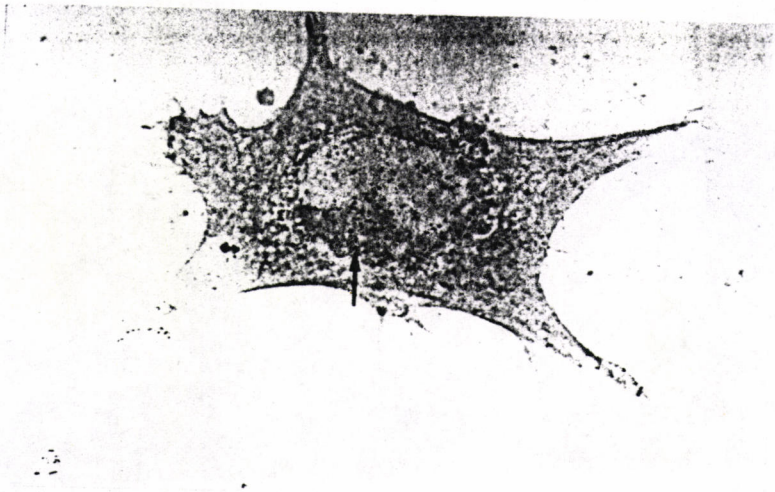

Fig. 5: Dark brown Immunoperxidase staining perinuclear intracytoplasmic inclusions (Arrow), at 24 hours p. i. (x1000).

REFERENCE:

1. Niven, S. F. Armstrog, J. A., Balfour, B. M., Kleperee, H. G. & Tyrrell, D. A. (1962). Cellular changes accompanying the growth of influenza virus in bovine cell cultures. J. Pathology Bacteriology. 84, 1-18.
2. Armstrong, C. L., Miranda, A. F. & Hus, K. C.(1978). Susceptibility of human skeletal muscle culture to influenza virus infection II.Cytopathology and immunofluorescence. J . Neurology Science. 35,43-57.
3. Saito, Y., Yashioka, I. & Igarashi, Y. (1970).Nuclear inclusions observed by electron microscope in cynomologus monkey kidney cells infected with influenza virus. Virology. 40, 408-409.
4. Archetti, I.,Bereezky, E, Rosati- Valente , F and Steve- Bocciarlh, D. (1970). Elongated structures present in cells infected with influenza virus. Archiv Virology. 29,275-286.
5. Alexander, D. J., collins, M. S. & Parkinson, M. (1982) Plaque forming ability in MDCK cells and structure of hemgglutinin of Influenza A viruses which differ in virulence for chickens. In proceedings of the first international symposium of avian influenza, 22-24 April 1981. Beltsville Maryland, USA 1981, pp. 148-156.
6. Alexander, D. J. (1986) Criteria for the definition of pathogenicity of avian influenza virus. In proceeding of the second international symposium on avian influenza, 3-5 Sept. 1986. Athens: USA, pp. 228-245.
7. Bosch, F. X., Orlich, M., Klenk, H. D. &Rott,R.(1979) The structure of the hemagglutinin A determinant for the pathogenicity of influenza viruses. Virology. 95, 197-207.

8. Ibrahim, H. M., Awang, I. P. R., Alexander, D. J., Manvell, R. J., Aini, I. & Ibrahim, A. L. (1990) Isolation of influenza viruses from passerine birds in Malaysia. Veterinary Record. 24, 582.
9. Ibrahim , H. M. (1993) In vitro and in vivo studies of Malaysian isolates of avian influenza virus. PhD thesis submitted to the Faculty of Veterinary Medicine and Animal Science, University Pertanian, Malaysia.
10. Hsiung, G. D. (1973) Diagnostic Virology; An Illustrated Hand book. (New Haven and London: Yale University Press).
11. Russell, p. H., Griffiths, P. C. & Cannon, M. J. (1983) A micro well immunoperoxidase test for screening hybridomas and for diagnosis NDV and Sendai Viruses. J. Immunological Methods. 61, 156-170.
12. Rott, R.C.(1981) The role of the hemagglutinin in infectivity and pathogenicity of avian influenza viruses. In proceeding of the first international symposium on avian influenza, 22-24 April 1981. Beltsville: Maryland, USA, pp. 116- 133.
13. Anisima, E., Ghendon, Y. & Markushin, S. (1980) Ultrastructural changes in cells induced by temperature-sensitive mutants of fowl plangue virus at permissive and non- permissive temperature. J. General Virology. 47,11-16.
14. Breithenfeld, P. M. & Schafer, W. (1957) The formation of fowl plague virus antigens in infected cells studied with flourescent antibodies. Virology. 4,328-345.
15. Grossberg, S. (1963) Cytocidal assay and propagation of human influeza viruses in cultred primary hamster kidney cells. Proceedings Social Experimental Biological Medicine. 113,546-553.

16. Blaskovic. P., Rhodes, A. J., Doane, F. W. & Labzoffsky, N. A. (1972) Infection of chick embryo tracheal organ cultres with influenza A2 (Hong Kong) virus I. Cytopathological, histopathological, immunofluorescence, hemadsorption and titration of released infections progeny. Archives Virology. 38,250-260.

17. Ciampor, F. M., Bystricka, M. & Rajcani, J. (1974) Ultrastructural localization of influenza vius antigens in infected chick embroy cells as revealed by a peroxidase-labelled antibody method Archives Virology. 46,341-352.

ألا مراضية الخلوية لفايروس أنفلونزا الطيور نوع $H_4 N_3$

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الخلاصة

تم إصابة طبقة كاملة من خلايا الزرع النسيجي المؤلفة من خلايا الاروماتات الليفية لجنين أفراخ الدجاج بفايروس انفلونزا الطيور المعزولة من الطيور المستأنسة . وفحص باستخدام صبغة الهيماتوكسلين والايوسين واختبار أنزيم البيروكسيد المناعي الغير مباشر لدراسة تأثير الفيروس على هذه الخلايا. تبين من خلال الدراسة بان التغيرات ألا مراضية الخلوية التي سببها الفيروس ظهرت بشكل مبكر على النواة وتضمنت ضخامة النواة والنوية للخلايا المصابة ، تهميش الكروماتين وظهور الأجسام الاشتمالية النواتية.أهم التغيرات الواضحة في هيولي الخلايا المصابة كانت ظهور الأجسام السايتوبلازمية المحتواة حول النواة وبعد 22 إلى 36 ساعة بعد الحقن كما ولوحظ وجود التحبب والتفجي في الخلايا المصابة بالفيروس. وضح اختبار أنزيم البيروكسيد المناعي تموضع المستضد الفيروسي في الخلايا المصابة حيث ظهر على شكل تفاعل قهوائي اللون في النواة أو الهيولي مشابهة لما لوحظ في صبغة الهيماتوكسلين والايوسين.