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NATURE OF POLYPEPTIDES SYNTHESIZED IN INFLUENZA A VIRUS-INFECTED NONPERMISSIVE CELLS OF THE IMMUNE SYSTEM

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Summary. — Inoculation with influenza A virus of nonpermissive cells from the immune system of Wistar rats was followed by an abortive reproduction cycle accompanied by the synthesis of viral polypeptides on the background of substantially decreased total protein synthesis. The de novo synthesized proteins in virus-infected immune system cells were shown to represent at least two types of viral protein, namely the nucleocapsid (NP) and matrix (M) proteins. The ability of influenza virus to partial expression of the genome in immunocytes along with the reversible injury to the cell metabolism should be taken into account in evaluating the possible mechanisms of action of influenza A virus on the functional activity of cells of the immune system.

Key words: *influenza A virus*; *protein synthesis*; *nonpermissive cells*; *immunocytes*; *polyacrylamide gel electrophoresis*

Introduction

Interaction with lymphocytes and macrophages of various viruses pathogenic for man or animals can be manifested in the form of a productive infection. If lymphocytes and macrophages represent a permissive system for the virus, their interaction may show a destructive or symptomless course of the biosynthesis of infectious particles. In nonpermissive cells, this process can be manifested by partial synthesis of individual structures which is not completed by the production of mature virions.

We showed (Polyak *et al.*, 1980; Dubrovina *et al.*, 1980*a*, *b*) that interaction of influenza virus with rat immunocytes represents a model of nonproductive infection characterized by the formation of individual virus-specific ribonucleoprotein (RNP) structures. Detection and identification of polypeptides synthesized de novo in virus-infected immunocytes is very important, because the appearance of viral proteins in infected macrophages and lymphocytes even in the absence of infectious virus formation may affect the character of the immunological reaction and the pathogenesis of the infection (Virelizier

et al., 1977; Brownson *et al.*, 1979; Smorodintsev, 1979, 1980; Dubrovina *et al.*, 1980*a*; Polyak *et al.*, 1980).

In the present work we studied the nature of polypeptides synthesized de novo in macrophages and splenocytes from Wistar rats 6 hr after inoculation with influenza A virus in comparison with the synthesis proceeding in susceptible dog embryo kidney (MDCK) cells.

Materials and Methods

Cells. Cultures of stable MDCK cells grown in Eagle's medium served as a susceptible system to influenza virus.

As models of immune system cells, we used suspension cultures of splenocytes and peritoneal exudate cells enriched by macrophages from non-stimulated Wistar rats (Dubrovina *et al.*, 1980*a*).

The cells were inoculated with an allantoic culture of influenza virus A/Leningrad/538/74 (H3N2).

Virus-specific polypeptide synthesis was investigated in cell suspension cultures in plastic tubes at a high multiplicity of infection [5—10 egg infection doses (EID) per cell]. Virus was left to adsorb in a minimal volume of Eagle's medium for 10 min at 37 °C.

The intensity of protein synthesis in uninfected and infected immune system cells was evaluated based on the level of incorporation of labelled precursors of protein synthesis into the acid-soluble fraction of the cells.

The inoculated cells were incubated for 4 hr in complete Eagle's medium which was then replaced by Eagle's medium devoid of 3 amino acids, namely leucine, glycine and alanine. The cells were incubated in the latter medium for 1 hr, after which ^{3}H-labelled leucine, glycine and alanine were added at a ratio of 37 kBq per ml and incubation was continued for 1 hr. Unadsorbed label was thoroughly washed by chilled phosphate buffered saline (PBS).

Identification of viral glycoproteins. At 4 hr after inoculation of the cells, ^{3}H-glucosamine (1.85 MBq per ml) was ad ded to the medium. One hr later, the excess label was washed off by chilled PBS. The cell pellet was kept at —20 °C until examined. Polypeptides labelled with radioactive amino acids or glucosamine were analyzed by electrophoresis in 7.5% polyacrylamide gel (PAGE) with 0.1% sodium dodecyl sulphate (SDS) according to Laemmli (1970).

Viral polypeptides were identified by immunoprecipitation. A formalin-inactivated suspension of *Staphylococcus aureus* (strain Cowan I) cells prepared according to Kessler (1975) was used as sorbent.

Specific antiserum to influenza virus was obtained by immunization of Chinchilla rabbits according to Etkind and Krug (1975). The titre of the antiserum used was higher than 1 : 40 000 in the haemagglutination inhibition test with 4 haemagglutination units of homologous virus.

For examination in the immunoprecipitation reaction, infected cells were lyzed according to Kessler (1975). The radioactivity of the samples used in this reaction was in the region of 10^5 counts/min. To obtain specific precipitate, 20—40 μl of specific antiserum were added to 200——400 μl lysate of the test material. The mixture was incubated for 15 min under continuous stirring, after which 100—200 μl of a 10% (w/v) suspension of the sorbent (inactivated *S. aureus*) were added. After 15 min, the complex of antigen, antibody and immunosorbent formed was removed by centrifugation at 2000 rev/min for 10 min. The sediment obtained was washed three times with 0.025 mol/l Tris-HCl buffer, pH 7.6, containing 0.005 mol/l EDTA and 0.05% Nonidet P-40, and suspended in 100—200 μl of a solution containing 6 mol/l urea and 4% SDS. Then followed hydrolysis in the presence of 50 mmol/l dithiothreitol for 3 min in a boiling water bath. The treated material was electrophoresed in 7.5% polyacrylamide gel in the presence of 0.1% SDS according to Laemmli (1970). Homogenate from infected cells similarly treated with normal rabbit serum and homogeneate from uninfected cells treated with specific serum served as controls.

Results

Comparison of total protein synthesis in influenza virus-infected and uninfected rat splenocytes and macrophages revealed a strong (up to 70%) inhibition at 5 hr after inoculation (Table 1).

Table 1. Comparison of protein synthesis in uninfected and influenza virus-infected immune cells

Cells	Exp. No.	Incorporation of labelled amino acids into the cells (counts/min) Uninfected	Infected	% inhibition of protein synthesis
Splenocytes	1	42 000	2 700	94
	2	18 000	6 607	63
	3	460	286	38
Peritoneal macrophages	1	2 828	1 222	57
	2	31 110	12 770	59
	3	15 000	2 000	87
	4	4 266	2 560	60

Radioactivity was determined 5 hr after inoculation of the cells. Cultures of immune system cells were inoculated with a dose of 5—10 EID_{50} per cell.

Comparative electrophoretic analysis of polypeptides in uninfected and virus-infected immunocytes confirmed disturbances in general polypeptide synthesis (Fig. 1). The electrophoretic pattern of polypeptides in infected non-stimulated splenocytes and macrophages and phytohaemagglutinin (PHA)-stimulated lymphocytes markedly differed from the characteristic distribution of viral proteins in infected MDCK cells, nor was it identical with the protein distribution pattern in uninfected cells.

We used two approaches to differentiate the synthesis of cellular polypeptides from that of viral polypeptides in infected immunocytes: 1) an analysis of the synthesis of glycoproteids, virus surface polypeptides, by their labelling with their carbohydrate precursor ^{3}H-glucosamine, and 2) identification of virion polypeptides labelled with radioactive (^{3}H)-amino acids by their precipitation with specific antiserum and further analysis in PAGE.

Analysis of ^{3}H-glucosamine-labelled polypeptides from virus-infected MDCK cells showed a characteristic peak of radioactivity that corresponded to the zone of non-dissociated haemagglutinin (Fig. 2.) In rat splenocytes, a similar zone of increased ^{3}H-glucosamine incorporation was not observed (Fig. 2), suggesting that a sufficient ammount of viral surface glycoproteids was not synthesized in these cells.

Virus-specific polypeptides in the lysate from infected immune system cells were demonstrated and identified by the immunoprecipitation method. To test the suitability of the selected conditions of immunoprecipitation with subsequent analysis of labelled antigens by PAGE, we checked the possibility of demonstrating influenza virus polypeptides labelled with radioactive (^{3}H)-amino acids which had been synthesized de novo in susceptible MDCK cells.

To bind viral antigens synthesized in the cells, lysate of an infected MDCK cell culture was mixed with homologous antiserum that had a titre of at least 1 : 40 000 in the haemagglutination inhibition test. The immune complexes formed were removed by adsorption onto *S. aureus* cells. Lysate of infected cells treated with normal rabbit serum and lysate of uninfected cells treated with immune serum served as controls. Materials bound to the corpuscular sorbent were subsequently subjected to PAGE (Fig. 3). Analysis of the test materials revealed in the experimental sample only 2 major and 2 minor peaks of radioactivity that corresponded to viral antigens.

A similar analysis of lysates of influenza virus-infected macrophages, splenocytes and lymphocytes (Fig. 3), in comparison with MDCK cells,

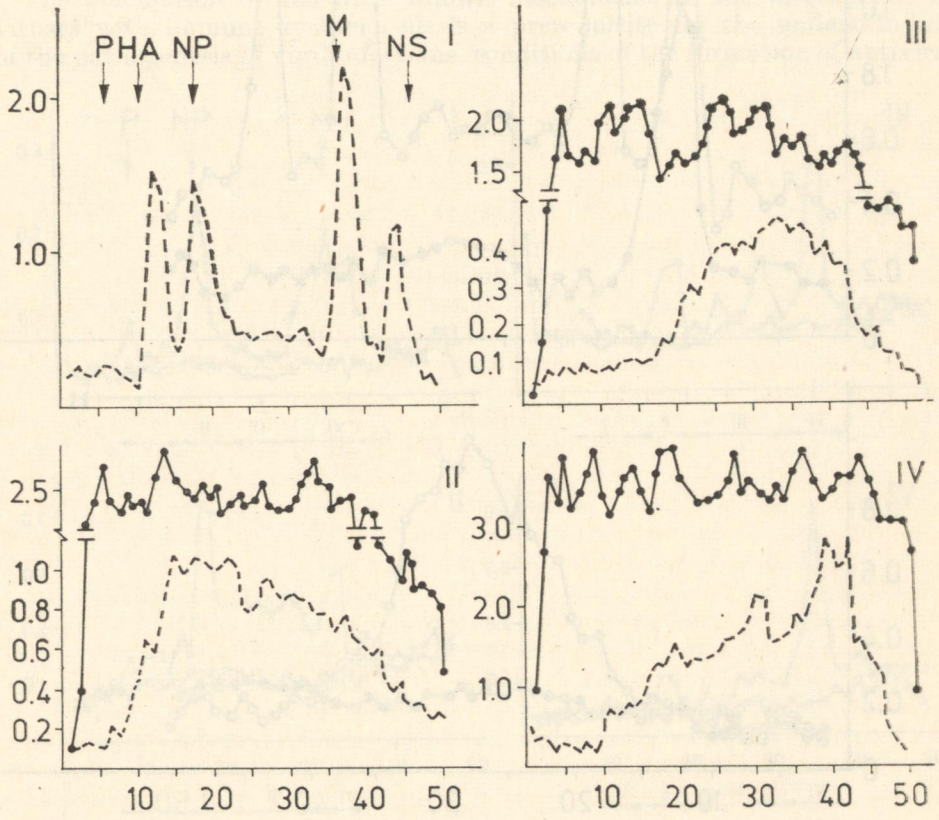

Fig. 1.

PAGE of polypeptides from influenza virus-infected MDCK cells (I), splenocytes (II), macrophages (III) and PHA-stimulated rat lymphocytes (IV)

Black circles: lysate of uninfected culture.

Broken line: lysate of infected culture.

Abscissa: number of gel segments. Ordinate: radioactivity of gel segments in counts/min $\times 10^{-3}$

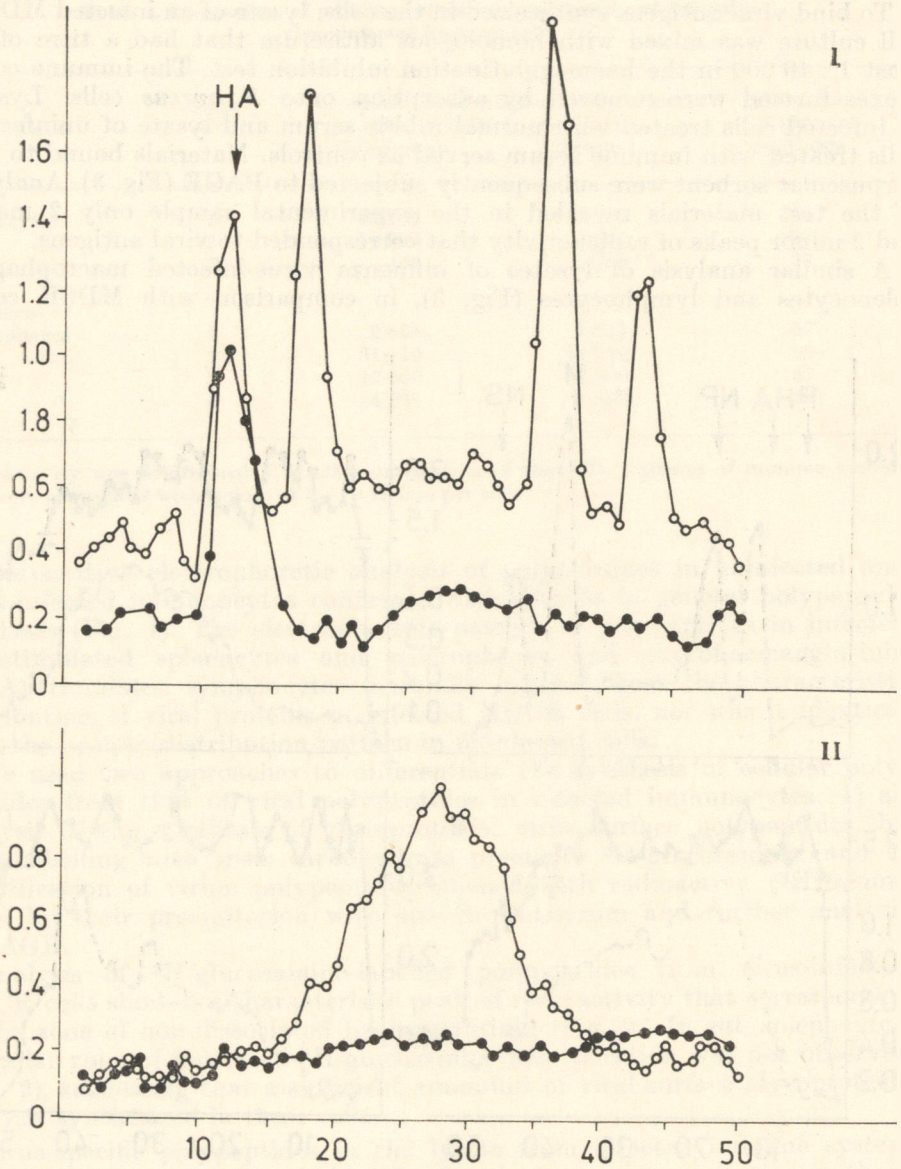

Fig. 2.

Identification of influenza A virus glycoproteins in MDCK cell culture (I) and rat macrophages (II)

Empty circles: lysate of cells labelled with radioactive (^{3}H) amino acids.
Black circles: lysate of cells labelled with ^{3}H-glucosamine.
Abscissa: number of gel segments. Ordinate: radioactivity of gel segments in counts/min × 1(

revealed a pattern of labelled viral polypeptides comparable to that found in MDCK cells. An especially marked increase in the amout of labelled material occurred in the zone of NP and M proteins. But it should be taken into account that, for the immunoprecipitation reaction, the lysates of susceptible MDCK cells and of immune system cells were adjusted to radioactivity range of about 10^5 counts/min. With immunocytes this required an about 10-fold higher amount of cell material. Examination of corresponding control lysates of immune system cells revealed no radioactive polypeptides with a similar electrophoretic mobility.

Discussion

The elucidation of the little known mechanisms of the interactions of viruses with immune system cells is a prerequisite for the understanding of the pathogenesis of viral infections, conditions of the formation of antiviral

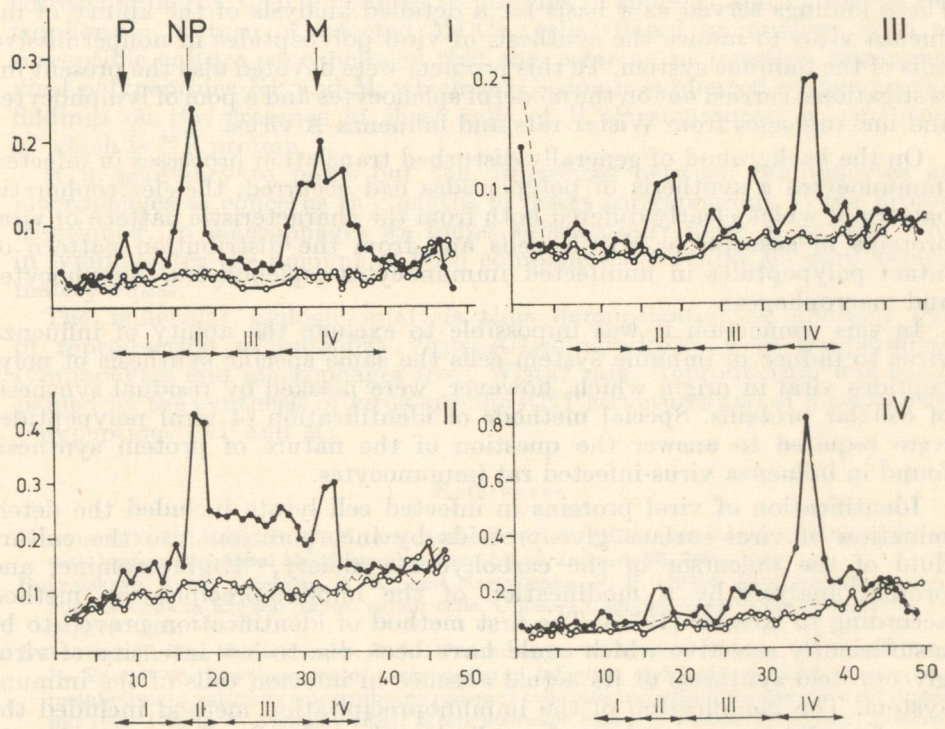

Fig. 3.
Comparative identification of virus-specific polypeptides in influenza virus-infected susceptible MDCK cells (I), lymphocytes (II), splenocytes (III) and macrophages (IV)
Immunoprecipitates obtained upon treatment of lysate of cells infected with: normal serum (○——○), immune serum (●——●).
Broken line: normal cells precipitated with immune serum.
Abscissa: number of gel segments. Ordinate: radioactivity of gel segments in counts/min × 10^{-3}

immunity and of the action of viruses on the immunological reactivity of the host organism (Smorodintsev, 1979, 1980; Polyak *et al.*, 1980). As an antigenic stimulant, the virus stimulates the development of an immunological reaction Being simultaneously an intracellular parasite, the virus may considerably modify the functional activity of immune system cells. Therefore, it is very important to determine whether the viral genome may be fully or partially expressed in these cells.

In accordance with our ideas and reports in the literature, influenza viruses partially express their genetic information in nonpermissive cell systems which do not suport the reproduction of complete virus (Polyak *et al.*, 1972; Dubrovina *et al.*, 1973; Kozeletskaya *et al.*, 1977; Bukrinskaya *et al.*, 1978; Polyak *et al.*, 1980). Cells of the immune system also proved to be a nonpermissive system for influenza virus in which, however, on the background of profound changes in the general metabolism, the synthesis of RNP virus structures was demonstrated (Polyak *et al.*, 1980; Dubrovina *et al.*, 1980a, b). These findings served as a basis for a detailed analysis of the ability of influenza virus to induce the synthesis of viral polypeptides in nonpermissive cells of the immune system. To this problem were devoted also the present investigations, carried out on the model of splenocytes and a pool of lymphocytes and macrophages from Wistar rats and influenza A virus.

On the background of generally disturbed translation processes in infected immunocytes a synthesis of polypeptides had occurred, the electrophoretic pattern of which clearly differed both from the characteristic pattern of viral proteins in susceptible MDCK cells and from the distribution pattern of intact polypeptides in uninfected immunocytes (splenocytes, lymphocytes and macrophages).

In this connection it was impossible to exclude the ability of influenza virus to induce in immune system cells the same specific synthesis of polypeptides viral in origin which, however, were masked by residual synthesis of cellular proteins. Special methods of identification of viral polypeptides were required to answer the question of the nature of protein synthesis found in influenza virus-infected rat immunocytes.

Identification of viral proteins in infected cell lysate included the determination of viral surface glycoproteids by incorporation into the culture fluid of the precursor of the carbohydrate moiety, ^{3}H-glucosamine, and protein analysis by a modification of the immunoprecipitation method according to Kessler (1975). The first method of identification proved to be insufficiently sensitive which could have been due to low intensity of virus glycoproteid synthesis or its actual absence in infected cells of the immune system. The modification of the immunoprecipitation method included the use of a solid immunosorbent, formalin-inactivated cells of *S. aureus* (strain Cowan I). The very high immunosorption potency of this microbial sorbent is due to the presence in its envelope of protein A (Kessler, 1975) which is able to bind intensively class G immunoglobulins. The use of a solid immunosorbent made the procedure of isolation of radioactive (^{3}H)-amino acid-labelled viral antigens in a complex with specific antibody from the cell

lysate easier, more rapid and more efficient. Pilot testing of the method in the susceptible MDCK cell culture made it possible to demonstrate positively 2 major and 2 minor peaks of specific viral radioactivity.

In similar investigations, Etkind and Krug (1975) found by this method 3 viral proteins, polypeptides P, NP and M, in a lysate of WSN virus-infected MDCK cells. In our experiment, the most marked zones of radioactivity in MDCK cells infected with the strain A/Leningrad/5/8/74 (H3N2) corresponded to NP and M proteins. The zone corresponding to P proteins was less marked. The remaining structural virion proteins were not observed, either due to their low concentration in the infected cell or due to the inherent losses of the method, corresponding to about 80% of the starting amount of labelled proteins.

By this mode of identification of virus-specific proteins synthesized in influenza virus-infected rat lymphocytes, splenocytes and macrophages, we were able to detect in a common pool at least two viral components, corresponding by their mobilities to proteins in zones II and IV on the electrophoretic pattern of infected MDCK cells. Based on analogy with the susceptible culture we concluded that the proteins in question represented viral polypeptides NP and M, which conclusion is supported by our previous findings on the presence in these cells of RNP structures, a constituent of which is NP protein.

It is necessary to point out the differences between lymphocytes and macrophages as concerns the heigths of peaks corresponding to the proteins mentioned. In macrophages we found substantially more M protein, while in lymphocytes the amount of both components (NP and M) were approximately equal.

The molecular biologic analysis thus demonstrated an abortive cycle of reproduction of influenza virus A/Leningrad/538/74 in nonpermissive lymphocytes and macrophages from Wistar rats. Reproduction was accompanied by the synthesis of virus-specific RNP structures and at least two viral polypeptides (NP and M).

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