Acta virol. **36:** 450-458, 1992

SIGNIFICANCE OF LONG LASTING PERSISTENCE OF INFLUENZA VIRUS ANTIGENS AT THE PORTAL OF INFECTION AND IN THE SPLEEN OF MICE

DUBROVINA, T. YA., IVANOVA, I. A., SHIDLOVSKAYA, N. K., POLYAK, R. YA

Institute of Experimental Medicine, Academy of Medical Sciences, Sankt Peterburg, C. I. S.

Received December 14, 1990

Summary. - Distribution of virus, its antigens and development of cellular factors of immunity were followed in the course of different forms of acute influenza virus infection in mice. Long term persistence of influenza virus antigens in the portal of entry and spleen were typical for of acute influenza. Lethal effect of influenza infection was caused by massive lesions induced by the virus and due to cell mediated immune response. The fate of infected individual seems to be decided during the first days of post-inoculation and depends on the ability of the virus to modify the cell membranes of the infected individual.

Key words: influenza virus; pathogenesis; viral antigens in the body; cell mediated immunity

Introduction

The outcome of virus infection is determined by hostparasite interactions. The penetrating virus provides a great variability of interactions with host cells, induces manyfold responses in eliminating the infectious agent which results in contraversial action of pathogenic factors (Ada *et al.*, 1981; Dubrovina, 1987).

To demonstrate the main pathogenic factors we selected modelling of different variants of acute infection and comparative analysis of complex parameters, reflecting virus reproduction and host defence in the kinetics of experimental influenza. We supposed that the antigen content at the portal of infection is important for further virus spread which is the result of the balance between virus reproduction capacity and the host defence. The first line of this defence is provided by nonspecific and then by specific immune responses (Smorodintsev and Polyak, 1970; Ada *et al.*, 1981; Polyak *et al.*, 1986; 1987). The diverse role of cell mediated immunity in the course of pathogenesis has been demonstrated (Ada *et al.*, 1981; Ada and Jones, 1986; Shidlovskaya, 1987). It was shown that the most massive consolidation in lung tissue coincided with the interval of most expressed activity of cellular responses in lungs and spleen (Mak

Nai Ki *et al.*, 1982). Synergism of the lethal effect of influenza virus and Streptococcus B at double infection coincides with the peak activity of natural killers (Dubrovina *et al.*, 1989). These observations allow to assume that postinfluenza pathology may be associated with the development of immune response (Ada and Jones, 1986).

In the present paper we compared the fate of virus, the occurence of its antigens (Ag) and the development of cell mediated immunity during lethal, sublethal and asymptomatic forms of infection.

Materials and Methods

We used the allantoic culture of repeatedly passaged pathogenic variant of influenza virus A/PR-8/34 (H1N1) and its avirulent its variant A/PR-8/59/1 (Egorov *et al.*, 1984).

Mice JI (CBA X C57, JASK) were inoculated by nasal route with stock virus in a dosis of 6.0 or 4.0 log EID_{50} (0.05 ml) and also with the ts-variant in the dose of 7.0 log EID_{50} (0.05 ml).

Infectious activity of the virus was measured by standard techniques on chick embryos (CE).

Influenza virus antigens were determined by dot immunobinding assay (Ivanova *et al.*, 1989), which was performed by blotting on nitrocellulose filter of 3 μl (1 μg/ml protein) cell lysate obtained after treating the organ suspension with 1 % solution of natrium deoxycholate and 2 % NP-40. The antigen was visualized by virus-specific antibody conjugated with horse-raddish peroxidase as described (Ivanova *et al.*, 1989). Paraphenylenediamine was used as substrate (0.13 mg/ml in 0.1 mol/l phosphate buffer pH 6.3 and 0.01 % H_2O_2). The Ag was quantified according to the surface of the stained blot measured at λ=633 nm on Ultrascan XL 2222-010 KV (LKB-Sweden).

Electrophoresis in PAG was made according to Laemmli (1970).

Viral proteins were identified by Western blotting (Towbin and Gordon, 1984) in the modification of Ivanova *et al.* (1989). The amount of organ suspension layered on the gel was standardized according to protein concentration. Noninfected tissue suspension was used as the control. After transfering to nitrocellulose paper the staining procedure was performed as described above.

Radiometric registration of cytotoxic lysis (CTL) was made as desribed by Rykova *et al.* (1981) and modified according to Shidlovskaya and Leonteva (1977) and Shidlovskaya (1989). Briefly, 0.8 MBq of ^{3}H-uridine ("Izotop" specific activity 962×10^3 MBq/mmol) was added to 4×10^6 cells. To test the natural killer activity (NK) we used K-562 cells as targets (Institute of Cytology, AMS U. S. S. R.). For testing virus-specific cytotoxicity (CTL) splenocytes were used from mice infected with the same virus as targets. Effector and target cells were mixed in a proportion of 1:100 then incubated for 4 hr in 5 % CO_2 atmosphere at 37 °C. The spleen cells were harvested as desribed (Malygin *et al.*, 1982). Erythrocytes were lysed by hopotonic shock and macrophages were removed by plastic adherence for 1 hr at 37 °C. The cells were cultured in modified medium 199 (Meshcheryakova *et al.*, 1979) containing 0.012 mol/l HEPES and gentamycine (0.001 mg/ml).

Mathematical analysis of results was performed on computer IBM-RS-AT by means of program "Immunoferm" in "Turbo-Pascale 4.0" language of the MS-DOS system (Voicekhovsky *et al.*, 1989).

Results

Modelling of experimental influenza in mice

Different course of influenza virus infection was established in mice with different dosis of the virulent A/PR-8/34 or its virulent ts-variant A/PR-8/50/I. After infection with log 6.0 EID_{50} of influenza A/PR-8/34 the lethality at 8 days p.i. surpassed 50 %, after inoculation of 4.0 log EID_{50} mortality did not exceed 50

by day 14 p.i. When 7.0 log EID_{50} of the ts-variant A/PR-8/59/I was inoculated, no mice died within 14 days p.i.

Comparison of virus growth and viral antigen detection in lungs and spleen

After infection with 6.0 log (high) and with 4.0 log (medium) inoculation dose infectious virus was found in lungs till 14 and/or 8 days p.i., and in the spleen till days 3–4 p.i., respectively (Figs. 1A and 1B). At the same time viral Ag was found in lungs as well as in spleen throughout the 28–37 days long observation period. The cyclic appearance of influenza Ag in the portal of entry was not dependent on the inoculation dose; the Ag content increased since days 2 and 5 p.i. At later postinfection intervals the amount of antigen, detected after inoculation of the medium virus dose, was higher than that after high inoculation dose; a second peak of Ag increase was observed at 8–9 days p.i.

During acute disease essentially similar kinetics of influenza virus Ag as in the lungs although in lower levels was also found in the spleen.

In contrast, in mice infected with the ts-variant A/PR-8/59/I, a strain with low reproduction capacity (Egorov *et al.*, 1984), the dynamics of antigen detec-

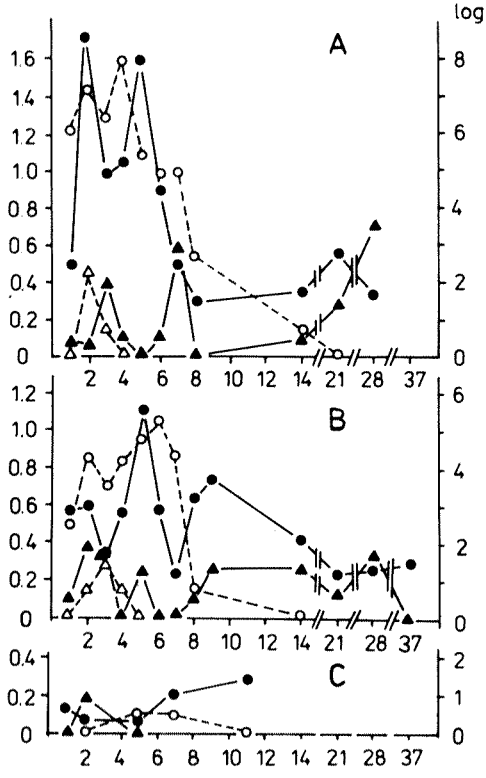

Fig. 1

Dynamics of the accumulation of infectious virus and distribution of its antigen in lungs and spleen of mice

A – lethat infection with 6.0 log EID_{50}

B – sublethal infection with 4.0 log EID_{50}

C – symptomless infection with 7.0 log EID_{50} ts-variant

Abscissa: observation days; ordinate: in the left – absorbancy at 633 mm in relative units; in the right – infections activity of the tested material in log EID_{50}.

Virus titres in lungs (○---○), spleen (△---△)

Antigen content in the lungs (● ●) and spleen (▲ ▲)

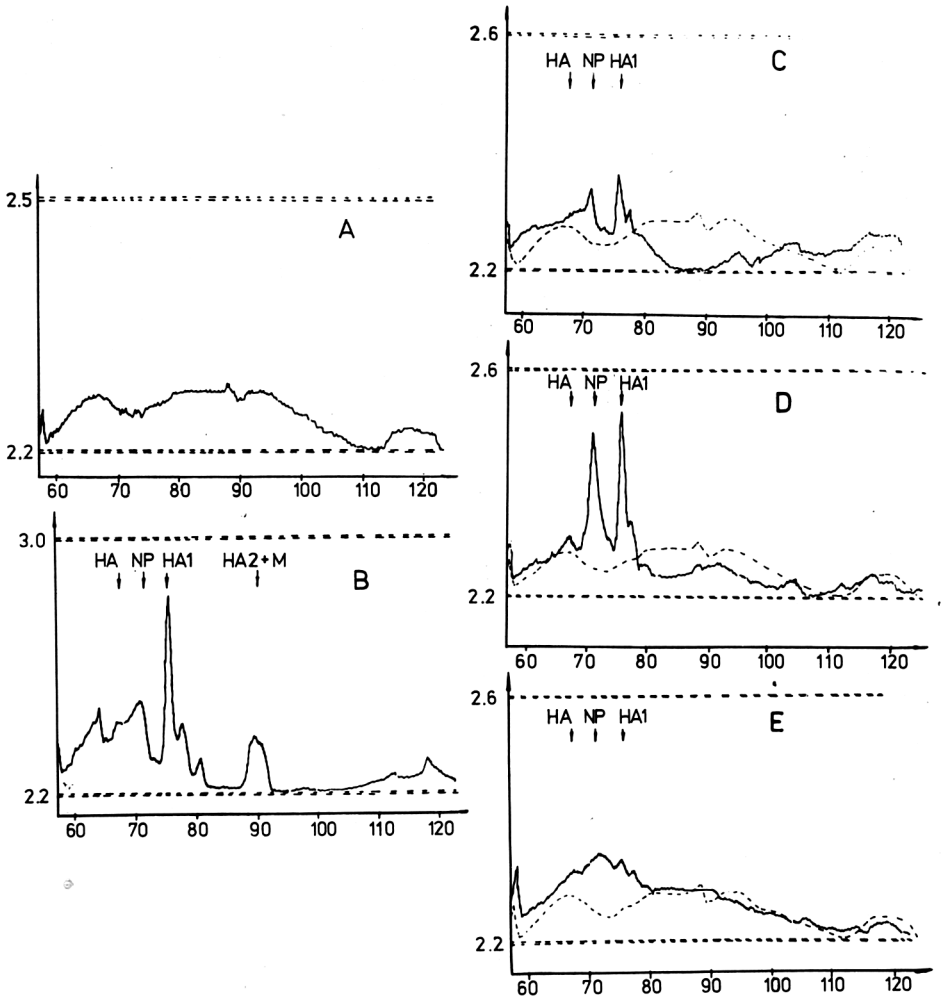

Fig. 2

Detection of influenza virus polypeptides in lungs at lethal and sublethal infections
Abscissa: distance from the start in nm; orginate: absorbancy at 633 nm
Double lines indicate the range of optical measurements.
Solid line: densitometric analysis of immunoblot results.
Interrupted line: control absorbancy (see also Fig. 1-A).
B - purified virus; C, D and F - lethal infection at 36 hr, 5 days and 21 days p.i. HA, HA1, HA2, M and NP indicated by arrows.

tion was different. Symptomless infection was accompanied by low but clear-cut antigen contents in lungs (Fig. 1C). The antigen content in lungs increased till days 7 to 11 p.i. No virus was found in, but Ag in spleen was found on day 2 p.i.

To confirm the specificity of viral Ag detection, specimens were examined by Western blot (Fig. 2-3). As compared to purified influenza virus, the investigated tissues revealed the presence of nucleoprotein (NP), uncleaved haemagglutinin (HA) and of the large HA-1 subunit. In sublethal infection the relative content of NP and HA-1 decreased by duration of the post-infection interval (Figs. 3A-D). The contemporarily occuring increase of uncleaved HA can be accompanied by nonproductive virus infection (Klenk *et al.*, 1977). During lethal infection in lungs the relative amounts of NP prevailed (Figs. 2B-2C).

In spleen, the relative content of individual polypeptides remained practically unchanged independently the severity of infection and of postinfection interval.

Dynamics of cellular immune response in experimental influenza.
The develop of cellular immune response was analyzed by means of specific

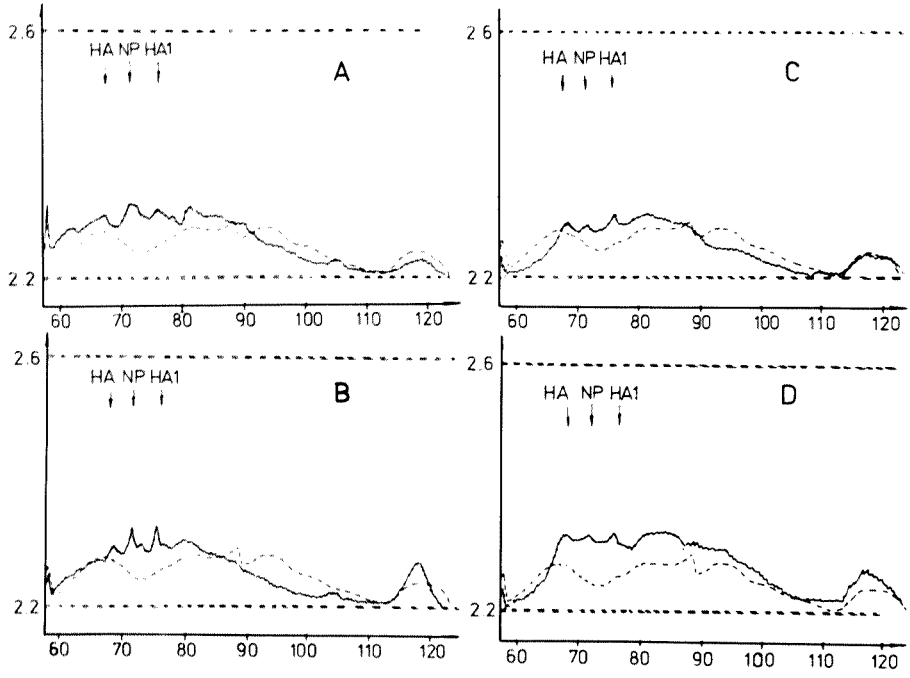

Fig. 3
Detection of polypeptides as described in Fig.2
A – D sublethal infection at days 2,5,9 and 28 p.i., respectively.

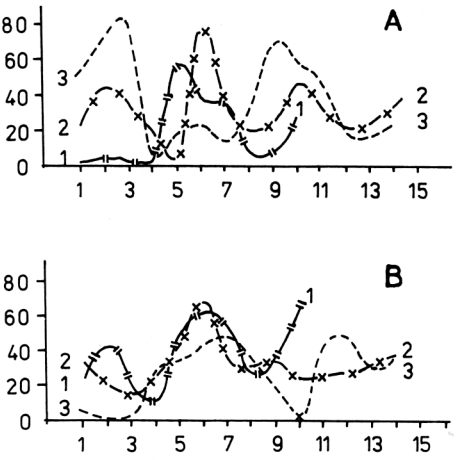

Fig. 4
Activity of normal killers (A) and virus specific T-lymphocytes (B) in experimental influenza
Abscissa - observation days: ordinate - cytotoxicity index (%) in lethal infection (1), sublethal (2) and symptomless (3) infections.

(CTL) and nonspecific (NK) cytotoxicity reactions. In all models a high level of NK and CTL activity was noted (Fig. 4). Interestingly, in mice infected with the ts-variant even small amounts of influenza Ag elicited a significant cell mediated response (Shidlovskaya *et al.*, 1989) as well as antibody formation (Egorov *et al.*, 1984). It should be noted that in lethal influenza no NK activity was found till day 4 p.i. (Fig. 4A), first reaction being observed on day 5 (Fig. 4B).

Analysis of the occurrence of virus specific target cells showed that cytolysis of infected splenocytes appeared in each infected mouse model studied, thus it was found not only in acute lethal forms but also in symptomless infection (Fig. 5). Following the dynamics of the formation of spleen target cells for CTL in nonlethal forms of infection it was found that their appearance was cyclic and transient with significant decrease to controls levels. In contrast, the lysis of target cells in the spleen in lethal forms of infection has remained extensive during all the observation period and had never returned to control levels.

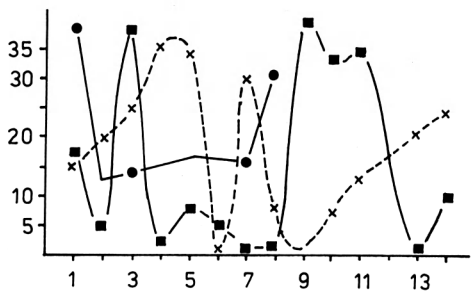

Fig. 5
Target cells for virus specific effector cells in the spleen of expermentally infected mice
Abscissa&: observation days; ordinate: cytotoxicity index at lethal (● ●), sublethal (x---x) and symptomless (■ ■) infections.

Discussion

Comparison of influenza virus infectivity assays and Ag content at portal of entry showed the presence of high levels of influenza Ag in both lethal and sublethal infections during the first 7–8 days p.i. and a long time persistence of this antigen although in lower concentrations. During the first period, the presence of Ag was associated with infectious virus and clinical disease, while during "clinical healing" virus infectivity disappeared despite of Ag persistence. The high Ag and infectivity levels in lungs decreased already within 3 days p.i. probably due to efficient virus elimination which was as intensive as its previous growth. During lethal infection no NK activity was detected until day 4 p.i., thus the main effector cells were those with cytotoxic activity. Under conditions of massive infection the activity of NK cells was probably blocked by their interaction with the virus itself. Influenza virus HA is known to depress NK cell cytotoxicity (Ali *et al.*, 1984). In addition, however, the absence of NK cell activity in spleen cells at early postinfection interval in lethal influenza may reflect the massive accumulation of these cells in lung tissue. During nonlethal influenza the most expressed NK activity in lung tissue appears at 48 hr p.i. (Ada and Jones, 1986).

Regardless to the sufficiently expressed cell mediated reactions and to the presence of other defence factors (Polyak *et al.*, 1986) the virus accumulated in a great amount between days 5 to 7 p.i. This lead to induction of specific and nonspecific effectors activity of which was especially high during acute experimental influenza between days 5 to 8 p.i. It is important that lethality culminated at the same interval. As the result of the high cell mediated response in this group by day 8 p.i. the Ag content was the lowest in comparison to surviving mice.

Data supporting the high activity of cellular defence at the time of peak lethality are interesting also in association with observations that cytotoxic effectors exert contradictory functions, i.e. not only prevention but they also act as factors of pathogenicity (Ada *et al.*, 1986; Askonas, 1988; Dubrovina, 1987). This was supported by recent results showing that cytotoxic cells itself did not induce pathologic changes in the absence of local lesions in symptomless infection or in sublethal infection if the lesions were ot large enough.

The prolonged presence of viral Ag in spleen deserves special attention. The induction of immune response is associated with a transient appearance of the Ag in the spleen of immunized animal (Kulberg, 1986). The prolonged persistence of viralAg in spleen can be explained by several processes. Influenza virus regardless of the state how it reaches the spleen, i.e. as free virus or in the form of immune complexes, when absorbed to spleen cells may alter their membranes. *In vitro*, influenza virus elicits abortive infection of immune system cells (Dubrovina, 1987), which may lead to expression of virus specific antigens at their surface and, in turn, modifies the membranes as result of the synthesis of virus specific proteins.

To elucidate to role of splenocyte lysis in influenza further studies are needed; elimination of Ag-labelled cells is especially high in lethal infection. The action of "splenocyte against splenocyte" might have a regulatory role by removing inducer cells after exerting their function. The increased lysis of splenocytes observed during lethal infection illustrates the transition of defence factors to pathogenetic ones. Similar regulatory mechanisms were described for Ag-presenting cells (Shyr-Te Yu *et al.*, 1986).

The cyclic character reflects the mutual dependence of these processes: of cells modification by the virus induces a cell mediated response which leads to elimination of specific Ag. If the activity of effector cells decreases, virus growth increases and more Ag is produced. Under such conditions the continuous presence of Ag seems a regular phenomenon in acute disease. The outcome of host infection depends on the amount of cells modified by the virus at most early postinfection intervals. If the extent of lytic reaction exceeds the intensity of the elimination of toxic products generated at lysis and the rate of regeneration of involved tissue, then recovery becomes more difficult or impossible.

References

Ada, G. L., and Jones, P. D. (1986): The immune response to Influenza infection. *Curr. Top. Microbiol. Immunol.* **128**, 1–54.

Ada, G. L., Leung, K. M., and Ertle, H. C. (1981): An analysis of effector T cell generation and function in mice exposed to influenza A or Sendai viruses. *Immun. Rev.* **54**, 4–24.

Ali, S. A., Rees, R. C., and Oxford, J. (1984): Modulation of human natural killer cytotoxicity by influenza virus and its subunit protein. *Immunology* **52**, 4, 687–695.

Askonas, B. A. (1988): T-cell diferentiation and effector function. *Immunology* **64**, suppl. 1, 51–52.

Dubrovina, T. Ya. (1987): Pathogenetic role of virus specific membrane modification in viral infections. In *Strategy of the Agent in Host Body*, pp. 87–96 (in Russian).

Dubrovina, T. Ya., Grabovskaya, K. B., Ivanova, I. A., Shidlovskaya, N. K., and Polyak, R. Ya. (1989): Lethal synergism of viral-bacterial infections (model: influenza – streptococci). *Vestn. Akad. Med. Nauk U. S. S. R.* No. **11**, 17–22 (in Russian).

Egorov, A. Yu., Medvedeva, T. E., Polezhaev, F. I., Alexandrova, G. I., and Ghendon, Yu. Z. (1984): Peculiarities of obtaining and characterization of a cold-adapted A/PR/8/34 influenza virus varian. *Acta virol.* **28**, 19–25.

Ivanova, I. A., Meringova, L. F., and Dubrovina, T. Ya. (1989): Detection of viral antigens in lungs and spleen of mice infected with influenza virus. *Molek. Genet. Microbiol. Virusol.* **No. 5**, 39–45 (in Russian).

Klenk, H.-D., Rott, R., and Orlich, M. (1977): Further studies on the activation of influenza virus by ptoteolytic cleavage of the hemagglutinin. *J. gen. Virol.* **36**, 151–161.

Kulberg, A. Ya. (1986): Regulation of Immune Response, *Meditsina, Moscow*, 223 p. (in Russian).

Laemmli, U. K., (1970): Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature (London)* **227**, 680–685.

Mak Nai-Ki, Zhang Yong-He, Gordon, L., Ada, G. I., and Tannock, G. A. (1982): Humoral and cellular response of mice to infectin with a cold-adapted Influenza A virus variant. *Inf. Immun.* **38**, 218–225.

Malygin, A. M., and Aprelikova, O. N. (1982): Natural antitumour activity of the splenocyte of C3HA mice, *Eksp. Onkol.* **4 (3)**, 37–39 (in Russian).

Meshcheryakova, I. E., Surkova, E. A., and Polyak, R. Ya. (1979): Transformation of mouse splenocytes stimulated with phytohaemagglutinin as related to the conditions of cultivation. *Citologiya* **21 (8)**, 982–986 (in Russian).

Polyak, R. Ya., Dubrovina, T. Ya., and Leonteva, G. F. (1986): Molecular and cellular basis of antiviral defence. In *Immunology, Actual Problems of Molecular Immunology*. Moscow, Itogi, Nauki i Tekhniki 17, 147–177 (in Russian).

Polyak, R. Ya., Belykh, L. M., Voicekhovsky, B. L., Demidova, S. V., Dubrovina, T. Ya., Dukhin, A. I., Zuev, S. V., Zueva, O. P., Ivanova, I. A., Kalyaev, D. V., Kramskaya, T. A., Leontieva, G. F., Lozinsky, V. P., Mazina, I. V., Mamontova, T. V., Meringova, L. F., Musheneva, M. S., Socheslo, L. I., Chetverikova, L. K., and Shindlovskaya, H. K. (1987): Systemic analysis of viral damage and the antiviral effect of chemotherapy in the influenza model, p. 10–25. In *Strategy of Infections Agent in The Host Body*. Leningrad (in Russian).

Rykova, M. P., Spirande, U. V., Zedgenidze, M. S., Lyakhov, V. V., and Fuks, B. B. (1981): A new and highly sensitive method of natural killer cell testing. *Immunologiya* No. **3**, 88–90 (in Russian).

Shidlovskaya, N. K. (1987): Formation of cellular immune response in virus infections, pp. 158–170. In *Strategy of Infections Agent in the Host Body*. Leningrad.

Shidlovskaya, N. K., and Leonteva, G. F. (1987): Use of mouse splenocytes as target cells for the analysis of cell mediated cytotoxicity in experimental influenza virus infection. *Vopr. Virusol.* **No. 2**, 241–244 (in Russian).

Shidlovskaya, N. K. (1989): Methodic approaches to Evaluation of Cellular Defence in Influenza (model splenocyte against splenocyte). *Zh. Mikrobiol. Epidem. Immunol.* **No. 7**, 96–100 (in Russian).

Shidlovskaya, N. K., Mazina, I. V., Dubrovina, T. Ya., and Polyak R. Ya. (1989): Cellular part of immune response and the kinetics of experimental infection elicited by influenza A virus of different properties. *Zh. Mikrobiol. Epidem. Immunol.* **No. 8**, 79–84 (in Russian).

Smorodintsev, A. A., and Polyak, R. Ya. (1970): Basis mechanisms of natural resistance to viral infections, pp. 3–19. In *Problems of Virology and Pathogenesis of Respiratory Virus Infections*. Leningrad (in Russian).

Shyr-Te Yu, Dekruyff, R. H., and Dore, M. E. (1986): Inducer T-cell-mediated killing of antigenpresenting cells. *Cell. Immunol.* **101**, 613–624.

Towbine, H., and Gordon, L. (1984): Immunoblotting and dot immunobinding-current status and outlook. *J. immunol. Meth.* **72**, 313–340.

Voicekhovsky, B. L., Meringova, L. F., Dukhin, A. I., and Agafonova, I. I. (1989): Investigation of general regularities of humoral immune response in virus infection by means of discrete analysis of the properties of specific immunoglobulins. *Vestn. Akad. Med. Nauk. U. S. S. R.* **No. 11**, 81–87 (in Russian).