# NATURAL INFLUENZA A VIRUS INFECTION OF MICE ELICITS STRONG ANTIBODY RESPONSE TO HA2 GLYCOPOLYPEPTIDE

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**Summary.** – Two influenza viruses, A/Dunedin/4/73 (H3N2) and A/Mississippi/1/85 (H3N2) were adapted to BALB/c mice. Groups of BALB/c mice were intranasally (i.n.) infected with either single dose of particular virus strain or successively with both virus strains and titers of serum antibodies against influenza virus antigens ("influenza virus antibodies") and those just against the HA2 part of hemagglutinin (HA) ("HA2 antibodies") were determined. Successive infection with virus strains Dunedin and Mississippi in interval of 21 days led to the strong increase of the proportion of anti-HA2 antibodies in sera, though whole antiviral titres remained in general unchanged. These observations confirmed that the HA2 glycopolypeptide (gp) part of influenza virus HA is very strong immunogen in natural infection.

**Key words:** antibody response; HA2 glycopolypeptide; influenza vaccine; mouse-adapted influenza virus; natural influenza infection

## Introduction

The specific antibody response to influenza viruses has been characterized in detail from various aspects. Particularly the biological significance of antibody response to HA1 gp (virus neutralization, hemagglutination-inhibition activity) has been characterized by several authors. Number and localization of major antigenic sites on globular part of HA as well as mapping of individual epitopes have been described (Wiley *et al.*, 1981; Wiley and Skehel, 1987; Wharton *et al.*, 1989).

HA2 gp was earlier not considered an important antigenic part of HA (Brand and Skehel, 1972; Eckert, 1973). The induction of specific HA2 antibodies has been demonstrated after intramuscular immunization of rabbits with intact influenza virus (Russ *et al.*, 1978; Brown *et al.*, 1980). More important was detection of antibodies specific to HA2 in human convalescent sera (Styk *et al.*, 1979a).

The HA2 part of HA is relatively antigenically stable (Both *et al.*, 1983) and shows a high intrasubtype sequence homology (Nobusawa *et al.*, 1991). Therefore it was not surprising that HA2 gp antibodies were cross-reactive. Graves *et al.* (1982) have even described cross-reactivity of HA2 polyclonal antibodies to subtypes H1 and H3. Intersubtype crossreactivity of some HA2-specific monoclonal antibodies was also documented (Kostolanský *et al.*, 1994; Varečková *et al.*, 2002). HA2 antibodies did not show any hemagglutination-inhibition or virus neutralization activities (Styk *et al.*, 1979b; Graves *et al.*, 1982; Becht *et al.*, 1984; Russ *et al.*, 1987; Sánchez-Fauquier, 1987). This is in agreement with the fact that he major role of the antibody-mediated virus neutralization is blocking the receptor-binding site located at the tip of HA molecule, on its HA1 part (Skehel and Wiley, 2000). When attachment of virus to cell receptor occurs, virus is endocyted and fused with the endosomal membrane to release the nucleocapsid into the cytoplasm. Acidic endosomal environment triggers irreversible conformational changes of HA leading to loss of the trimeric structure of the HA1 part (Skehel *et al.*, 1982; Ruigrok *et al.*, 1986) and refolding of the HA2 part which is accompanied by changes in its antigenic properties, including emerging of the

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**Abbreviations:** aa = amino acid; ELISA = enzyme-linked immunosorbent assay; gp = glycopolypeptide; HA = hemagglutinin, hemagglutination; HAU = HA unit; i.n. = intranasally; MP = matrix protein; NP = nucleoprotein; p.i. = post infection; PBS = phosphate-buffered saline

previously hidden epitopes (Yewdell *et al.*, 1983; Daniels *et al.*, 1983; Webster *et al.*, 1983; Russ *et al.*, 1987; Kostolanský *et al.*, 1988; Varečková *et al.*, 1993). Most recently, Varečková *et al.* (2003), using a panel of monoclonal antibodies specific to HA2, have described a fusion-inhibition activity of some of them, especially of those recognizing the fusion-active N-terminus of the HA2 gp or binding in close proximity to it.

The aim of this study was to investigate further the immunogenicity of the HA2 gp in natural infection, using the mouse model. We measured levels of influenza virus antibodies and HA2 antibodies after single or repeated intranasal infection of mice with mouse-adapted influenza viruses of different virulence.

## Materials and Methods

*Viruses.* The following influenza virus strains were used: A/Dunedin/4/73 (H3N2) and A/Mississippi/1/85 (H3N2), both from Institute of Virology, Bratislava, Slovak Republic, and A/Sydney/5/97 (H3N2) from National Institute for Medical Research, London, UK. The virus strains were propagated in fertilized chicken eggs and purified from allantoic fluids by sucrose gradient centrifugation (Russ *et al.*, 1974).

*Recombinant EHA2 polypeptide* (amino acids (aa) 23–185) from the A/Aichi/2/68 (H3N2) virus strain expressed in *E. coli*, was prepared as described by Chen *et al.* (1995).

*Adaptation of influenza viruses to mice.* Adaptation was performed on 6-week-old BALB/c mice. In the first step, 60 µl of allantoic fluid containing infectious virus was i.n. applied to nostrils of a mouse under light ether anesthesis. After 48 hrs the mouse was lethally anesthesized, lungs excized, homogenized in 2 ml of cold phosphate-buffered saline (PBS) and clarified by centrifugation. The supernatant (60 µl) was immediately applied i.n. to another mouse. Lethally-adapted strains Dunedin and Mississippi were obtained after 13 and 12 lung passages, respectively. After the final passage the adapted strains were propagated to appropriate amounts in chicken embryos and the infectious allantoic fluids were saved and their HA and infectious titers were determined. The stock allantoic fluids were aliquoted and stored at -70°C.

*Determination of infectious titer ($LD_{50}$) in BALB/c mice.* Groups of four 6-week-old BALB/c mice were i.n. infected with twofold dilutions of a virus in PBS pH 7.2 in a volume of 60 µl per mouse. Mice were anesthesized with ether before virus inoculation. The survival rate in each group was observed for 14 days. $LD_{50}$ was determined in a standard way.

*Repeated infection of BALB/c mice.* Groups of mice were i.n. infected successively with the two strains in a sequence Dunedin→Mississippi or Mississippi→Dunedin respectively, according the following scheme.

Day 0, group 1: 8 mice infected with 0.38 $LD_{50}$ of Dunedin.

Day 0, group 2: 8 mice infected with 0.05 $LD_{50}$ of Mississippi.

Day 21, group 1a: 4 mice reinfected with 0.05 $LD_{50}$ of Mississippi.

Day 21, group 1b: 4 mice reinfected with 0.45 $LD_{50}$ of Mississippi.

Day 21, group 2c: 4 mice reinfected with 0.38 $LD_{50}$ of Dunedin.

Day 21, group 2d: 4 mice reinfected with 1.01 $LD_{50}$ of Dunedin.

Each mouse received a strain in a volume of 60 µl, using PBS as diluent. Mice were bled 14 days after the second immunization dose. The sera were stored at -20°C.

*ELISA.* 300 ng of purified virus or 250 ng of purified recombinant EHA2 polypeptide was adsorbed onto each well of a microtitration plate. The adsorbed virus was then exposed to either pH 7.2 or 5.0 by adding the McIlvaine buffer for 30 mins. After saturation with 2% nonfat dry milk in PBS, individual sera in various dilutions were added and the plates were incubated for 90 mins. The reaction was detected by adding a swine anti-mouse IgG conjugated with peroxidase (SEVAC, Czech Republic) and an enzyme substrate (o-phenylene-diamine with hydrogen peroxide). After stopping the color development by adding 3 M HCl $A_{492}$ was measured. Titers were expressed as reciprocal values of serum dilutions possessing the absorbance over 0.5 after subtraction of the background.

## Results and Discussion

*Determination of $LD_{50}$ dose of mouse-adapted influenza virus strains*

For i.n. infection of mice two influenza virus strains adapted to mice were used: A/Dunedin/4/73 (H3N2) and A/Mississippi/1/85 (H3N2). After adaptation, both viruses were titrated on 6-week-old BALB/c mice to determine their $LD_{50}$. For the Dunedin strain, the 50% lethality for mice was obtained with a dose of 5 HA units (HAU). The Mississippi strain caused the 50% lethality of mice in 16 days post infection (p.i.) with a dose of 0.06 HAU. Titration of HA activity of purified preparations of both strains showed that 1 HAU of each strain represents approximately the same amount, about 20 ng. Thus the Mississippi strain was approximately 90-times more virulent for BALB/c mice than the Dunedin strain. The reason for this difference is unknown.

*Influenza virus antibodies in sera after immunization of mice with a single dose*

To follow the specific antibody response in mice after i.n. infection, we used different inoculation doses of both viruses, starting from 1.3 (Dunedin) or 2.8 (Mississippi) $LD_{50}$. The sera, collected on day 14 p.i. from animals in particular group were pooled and examined by enzyme-linked immunosorbent assay (ELISA) for determination of titers of influenza virus antibodies and HA2 antibodies. The influenza virus antibodies were determined using a purified homologous virus adsorbed onto wells and pretreated with PBS pH 7.2 or McIlvaine buffer pH 5.0. It should be stressed that after adsorption of a virus strain onto wells antibodies specific both to HA as well as to internal virus proteins, mainly NP and M1, were able to bind to them. Results of

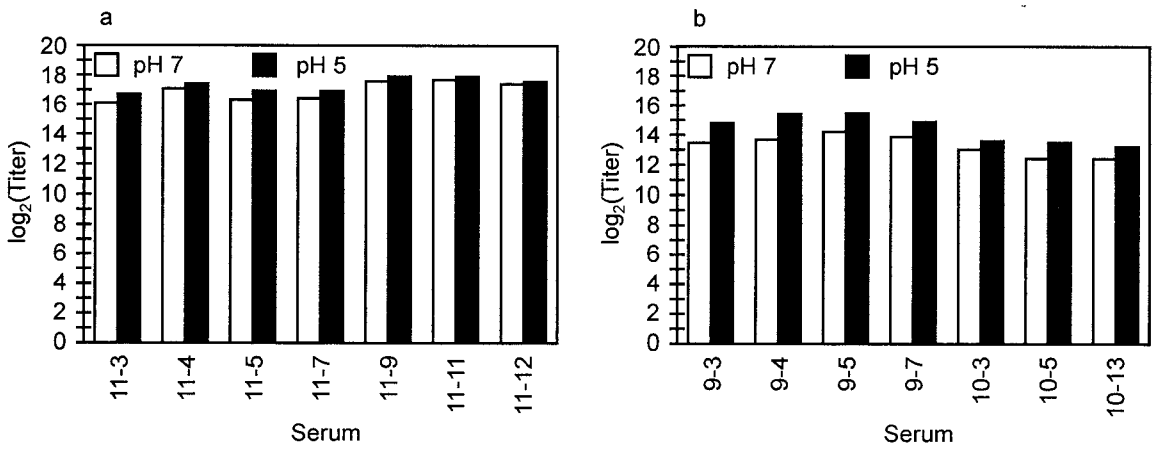


**Fig. 1**
**Dunedin (a) and Mississippi (b) antibodies after single infection**
Virus doses applied are given in Tables 1 and 2.

determination of titers of antibodies by ELISA are shown in Figs. 1a and 1b.

From the values obtained it follows that mice infected with influenza virus Dunedin strain developed influenza antibodies with titers ranging from 70,400 to 248,000 (Table 1). Interestingly, this titer in a given range is not in any correlation with the infectious dose used. On the contrary, the highest dose of virus (the serum pool 11-3) led to a relatively small antibody titer. Western blot analysis of these sera revealed presence of antibodies specific to HA as well as NP and M1 protein independent on the dose of infectious virus used (data not shown). According to this observation it seems that fully developed antibody response can be elicited even by a very small virus dose in natural infection.

The influenza virus antibody response to a single infection dose of the Mississippi strain also resulted in significant titers within a broad range of i.n. doses (Fig. 1b, Table 2). In contrast to the Dunedin strain, the Mississipi strain appeared to be less efficient inducer of antibody response, as the serum pool originating from the latter strain showed generally lower titers of antibodies than that originating from the former one. The reason for this observation is unclear. It is not likely that this difference is related to that in virulence since the applied virus dose ranges were overlapping. The Western blot analysis also proved the presence of antibodies against the internal NP and M1 proteins in sera of infected mice (data not shown). Elicitation of antibodies against internal virus proteins in natural infection is expectable (Sukeno *et al.*, 1979; Joassin *et al.*, 1983; Khristova *et al.*, 1988).

**Table 1. Titers of sera after single infection with the Dunedin strain**

| Serum No. | No. of mice | Infection dose ($LD_{50}$) | Antibody titers detected with | | |
|---|---|---|---|---|---|
| | | | Dunedin pH 7 | Dunedin pH 5 | Recomb. EHA2 polypeptide |
| 11-3 | 1 | 1.3 | 70,400[a] | 107,000 | 2,720 |
| 11-4 | 4 | $6.3 \times 10^{-1}$ | 145,000 | 176,000 | 1,910 |
| 11-5 | 4 | $3.1 \times 10^{-1}$ | 78,000 | 126,000 | 2,570 |
| 11-7 | 4 | $7.9 \times 10^{-2}$ | 87,600 | 126,000 | 1,440 |
| 11-9 | 2 | $2.0 \times 10^{-2}$ | 204,000 | 248,000 | 2,730 |
| 11-11 | 2 | $4.9 \times 10^{-3}$ | 215,000 | 246,000 | 2,800 |
| 11-12 | 2 | $2.5 \times 10^{-3}$ | 173,000 | 198,000 | 2,500 |
| Average | | | 139,000 | 175,286 | 2,381 |

[a]Titers are expressed as reciprocal values of serum dilutions having $A_{492} = 0.5$ after subtraction of the background. The values were calculated as averages from at least two measurements by linear interpolation for absorbance $A_{492} = 0.5$. Non-immune serum at lowest dilution exhibited absorbance values below the background + 3 SD.

**Table 2. Titers of sera after single infection with the Mississippi strain**

| Serum No. | No. of mice | Infection dose ($LD_{50}$) | Antibody titers detected with | | |
|---|---|---|---|---|---|
| | | | Mississippi pH 7 | Mississippi pH 5 | Recomb. EHA2 polypeptide |
| 9-3 | 1 | 2.8 | 11,200[a] | 29,400 | 632 |
| 9-4 | 2 | 1.4 | 13,600 | 42,700 | 1,160 |
| 9-5 | 3 | $7.1 \times 10^{-1}$ | 18,300 | 46,400 | 1,920 |
| 9-7 | 4 | $1.8 \times 10^{-1}$ | 15,000 | 30,000 | 619 |
| 10-3 | 2 | $4.4 \times 10^{-2}$ | 8,140 | 12,100 | 772 |
| 10-5 | 2 | $1.1 \times 10^{-2}$ | 5,490 | 11,900 | 1,100 |
| 10-13 | 2 | $1.4 \times 10^{-3}$ | 5,450 | 9,660 | 1,410 |
| Average | | | 11,026 | 26,023 | 1,088 |

[a]For the legend see Table 1.

Both sera induced by Dunedin as well as those induced by Mississippi revealed markedly increased binding to the homologous virus after the pH 5 treatment (Figs. 1a and 1b, Tables 1 and 2). An increased binding to the low pH-modified virus was less apparent in sera against Dunedin; the difference in reaction with the pH-influenced and native virus was about 30%. The sera obtained after infection with Mississippi revealed a very high preference of binding to the pH 5-modified virus, by even more than 100% as compared to the native antigen. The increased reactivity with the acidified virus resulted most probably from conformational changes of viral HA accompanied by an increased accessibility of the HA2 part of HA to binding of the antibodies. The binding of antibodies to internal virus antigens (NP and M1 protein) was not influenced by exposure of the adsorbed virus to pH 5.0 (E. Varečková, unpublished data). Therefore the increased reactivity of sera to the acidified virus can be attributed to conformational changes of HA, i.e. to the improvement of accessibility of its HA part.

*Influenza virus antibodies in sera after immunization of mice with two successive doses*

Next we addressed the question whether a repeated exposure to antigen during the second natural infection will trigger further increase of the antibody titer. To overcome the immunity induced by the first infection with Mississippi or Dunedin another antigenically distinct H3N2 strain (Dunedin or Mississippi) was used for the second infection.

For the second infection two different virus doses were used (see Materials and Methods and Table 3).

The sera obtained from mice in individual groups were pooled and examined by ELISA with the purified strains Dunedin and Mississippi, both at neutral- and pH 5.0-treated form (Figs. 2a and 2b; Table 3). Similarly to the single infection (Figs. 1a and 1b), all sera after the repeated infection revealed a markedly preferential binding to the strain treated with acidic pH (Figs. 2a and 2b). In fact, the reactivity of the sera with the acidified antigen increased even more, up

**Table 3. Titers of sera after successive infection with the strains Dunedin and Mississippi**

| Serum No | Primary infection with Dunedin Infection dose ($LD_{50}$) | Secondary infection with Mississippi Infection dose ($LD_{50}$) | Antibody titers detected with | | | | |
|---|---|---|---|---|---|---|---|
| | | | Dunedin | | Mississippi | | Recomb. EHA2 polypeptide |
| | | | pH 7 | pH 5 | pH 7 | pH 5 | |
| 12-1a | 0.38 | 0.05 | 73,500[a] | 146,000 | 851 | 7,200 | 40,700 |
| 12-1b | 0.38 | 0.45 | 23,000 | 67,200 | 930 | 3,850 | 17,400 |

| Serum No | Primary infection with Mississippi Infection dose ($LD_{50}$) | Secondary infection with Dunedin Infection dose ($LD_{50}$) | Antibody titers detected with | | | | |
|---|---|---|---|---|---|---|---|
| | | | Dunedin | | Mississippi | | Recomb. EHA2 polypeptide |
| | | | pH 7 | pH 5 | pH 7 | pH 5 | |
| 12-2c | 0.05 | 0.38 | 18,700 | 100,000 | 98,800 | 127,000 | 47,500 |
| 12-2d | 0.05 | 1.01 | 44,200 | 164,000 | 63,200 | 156,000 | 35,900 |

[a]For the legend see Table 1.

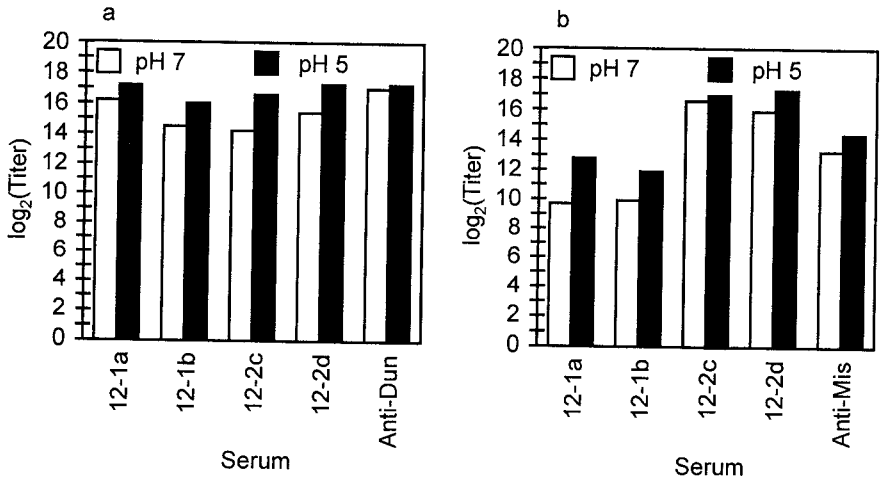


**Fig. 2**
**Viral antibodies after repeated infection**

Antibodies detected with virus Dunedin (a) or Mississippi (b).
Anti-Dun – columns represent average titre of sera after single i.n. infection with Dunedin (see Table 1).
Anti-Mis – columns represent average titre of sera after single i.n. infection with Mississippi (see Table 2).
Virus doses and infection sequence for preparation of immune sera are given in Table 3.

to 5-times, as compared to those obtained after a single immunization, when examined with Dunedin (Fig. 2a, Table 3), particularly the serum No. 12-2c.

Unexpected properties possessed the sera 12-1a and 12-1b, obtained from mice after the repeated immunization with viruses in the sequence Dunedin-Mississippi. These sera had a very weak reactivity with Mississippi (Fig. 2b and Table 3), reaching titers below 10,000 or 1,000, respectively (strain treated at pH 5.0 or 7.0, respectively). However, a significant binding of these sera (12-1a and 12-1b) appeared when examined with Dunedin, having titers comparable to those of the sera 12-2c and 12-2d (Fig. 2a and Table 3). It is evident that this binding activity belonged to the population of antibodies markedly specific to Dunedin. Despite a narrow strain (i.e. Dunedin) specificity of sera 12-1a and 12-1b, these antibodies showed a remarkably increased binding to the acidified virus. However, the antibodies binding to the acidified virus should not be strain-specific; in fact they are cross-reactive. This observation may be explained by the phenomenon of so called original antigenic sin. It means that production of the antibodies induced by the primary infection with the Dunedin strain was enhanced by the subsequent infection with the Mississippi strain. Simultaneously, the Mississippi antibodies (strain-specific) were produced in low titers as this strain is a poor immunogen.

Comparison of serum titers after single and repeated infection showed that influenza virus antibody level in sera with Dunedin antibodies did not increase when mice were infected repeatedly (Fig. 2a and Table 3). Some raise of titers in the sera obtained from repeated infection could be followed only in comparison with the average titer in sera obtained after single infection with the Mississippi strain (Fig. 2b and Table 3, the sera 12-2c and 12-2d). In other cases, the repeated infection did not lead to an increase in titers of influenza virus antibodies as compared to the single infection.

*HA2 antibodies in sera after single and repeated i.n. immunization of mice*

Selected sera obtained after single and successive i.n. infection were examined by ELISA for determination of the level of HA2 antibodies specific to the HA2 part of viral HA. In these experiments, the recombinant EHA2 polypeptide expressed in *E. coli*, originated from the light chain of HA of the influenza virus A/Aichi/2/68 (H3N2), was used as a detector antigen (EHA2). In comparison with viruses used as antigens, the recombinant EHA2 polypeptide was not treated with a low pH before reaction. Based on our experimental data (not shown) we concluded that the HA2 epitopes after adsorption of the recombinant to the solid phase are accessible to HA2 antibodies and exposure of this antigen to low pH does not further change its conformation since EHA2 is already folded into the low pH conformation (Chen *et al.*, 1995). As the antigenicity of the HA2 part of HA remains unchanged between the strains belonging to the same (H3) subtype, ELISA with EHA2 allowed detection of HA2 antibodies in both the Dunedin and Mississippi antisera.

From the results obtained it follows that the sera after a single i.n. infection with mouse-adapted viruses have different titers of HA2 antibodies and, there is no apparent

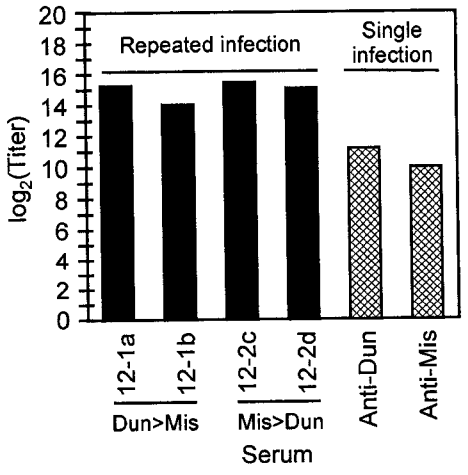


**Fig. 3**
**HA2 antibodies after repeated infection**

Hatched columns represent average titers of sera after single i.n. infection with Dunedin (see Table 1) or Mississippi (see Table 2). Virus doses applied are given in Table 3.

correlation between the titer of HA2 antibody and the dose of virus applied i.n., Dunedin or Mississippi (Tables 1 and 2). However, in general, lower titers of HA2 antibodies in the sera obtained after a single dose of the Mississippi strain coincided with overall lower titers of influenza virus antibodies.

The most interesting is a finding that the repeated i.n. infection induced relatively very high titers of HA2 antibodies (Fig. 3 and Table 3). Such a dramatically increased proportion of HA2 antibodies was present in all the sera examined despite succession of different strains in repeated infections (Dunedin-Mississippi and Mississippi Dunedin) or different doses used for the second infection. Absolute values of HA2 antibody titers in individual sera ranged from 17,400 (the serum 12-1b) to 47,500 (the serum 12-2c) (Table 3). A relative increase of HA2 antibody titers, assuming average titers after single infection 2,381 for Dunedin and 1,088 for Mississippi as reference values, was 17-fold for the serum No. 12-1a related to single infection with Dunedin, 7-fold for the serum No. 12-1b related to single infection with Dunedin, 44-fold for the serum No. 12-2c related to single infection with Mississippi, and 33-fold for the serum No. 12-2d related to single infection with Mississippi. Here, it must be emphasized that such increases do not correspond to the titers of influenza virus antibodies, which remained in fact unchanged, without any booster effect of a repeated exposure to virus.

In conclusion, the presented results show that natural infection with influenza virus leads to production of antibodies specific to the surface and internal viral antigens. Such a qualitatively equal antibody response is induced after infection with very broad range of infectious doses. Our experiments suggest that even a very small dose representing e.g. one thousandth of the lethal dose for mouse could induce sufficient level of antibody response needed for protection from subsequent infection of antigenically similar strain. When this conclusion is extrapolated to the human population it suggests that during "average" influenza epidemic there might be certain proportion of population inaparently infected and these individuals develop effective immune response. A repeated natural infection may preferentially increase antibody response to viral antigens which are not directly involved in virus neutralization. Particularly, our experiments showed that the HA2 part of influenza virus HA is very strong immunogen during natural infection of mice. We suppose that this observation might be valid also for humans. It could be expected that human sera after influenza primary infection (i.e. sera from very small children) contain low levels of HA2 antibodies in contrast to those in adults. Nevertheless, it should be reminded that a possible contribution of HA2 antibodies to the course of infection is difficult to address and thus still unclear. It will be important to observe the virus neutralization activity of mouse influenza virus antibodies/antisera, obtained with different doses of infectious virus, on permissive cells *in vitro* as well as *in vivo* in protection experiments on mice with passively transferred serum with the aim to determine whether the virus neutralization activity of mouse antisera corresponds to the titer of their influenza virus antibodies. This should answer the question whether a very small dose of infectious influenza virus causing an inapparent infection can induce an immune response sufficient for effective protection from disease.

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