Haemagglutination inhibition (HI) assay

Serum HI titres were quantified using whole virion influenza virus solution and chicken erythrocyte suspension. In a plate, 25 μl of 1:4 dilution of serum was serially twofold diluted. Then 25 μl of solution containing 8 HA units of whole virion influenza virus and 25 μl of 0.5% chicken erythrocyte suspension were added. HI antibody titres were expressed as the reciprocal of the highest dilution of the serum of the wells exhibiting haemagglutination inhibition. The lowest titre was 16. It should be remembered that expression of the dilution of sera in Japan is different from that in the US where the original dilution of serum is used as the titre. For example, a dilution of 1:16 in the US corresponds to a dilution of 1:64 in Japan.

Statistics

In both mucosal IgA antibody levels and serum HI antibody levels, fourfold or greater increases were considered significant. The χ^2 test with Yates' correction was used for statistical analysis.

RESULTS

Figure 3 depicts the serum HI antibody responses to A/Beijing H3N2. *Figure 3a* and *b* show responses of volunteers who received live vaccine first and inactivated vaccine second. In total, 23% (*Figure 3a*) manifested fourfold or greater increases in serum HI levels after administration of live vaccine, and 14% (*Figure 3b*) manifested fourfold or greater increases after administration of inactivated vaccine. *Figure 3c* and *d* show responses of volunteers who received inactivated vaccine first and live vaccine second. As many as 65% (*Figure 3c*) manifested fourfold or greater increases in serum HI titre after inactivated vaccine while none (*Figure 3d*) showed serum HI antibody responses to live vaccine. In the group who received live vaccine first (*Figure 3a*) 22/35 had preimmunization serum HI levels of $\leqslant$64 and eight of the 22 (36%) showed fourfold or greater increases in serum HI levels after administration of live vaccine. None of the volunteers who had preimmunization HI levels of $\geqslant$128 responded to the live vaccine. In the group who received inactivated vaccine first (*Figure 3c*) 29/40 had preimmunization serum HI levels of $\leqslant$64 and 21 of these 29 (72%) showed fourfold or greater increases in

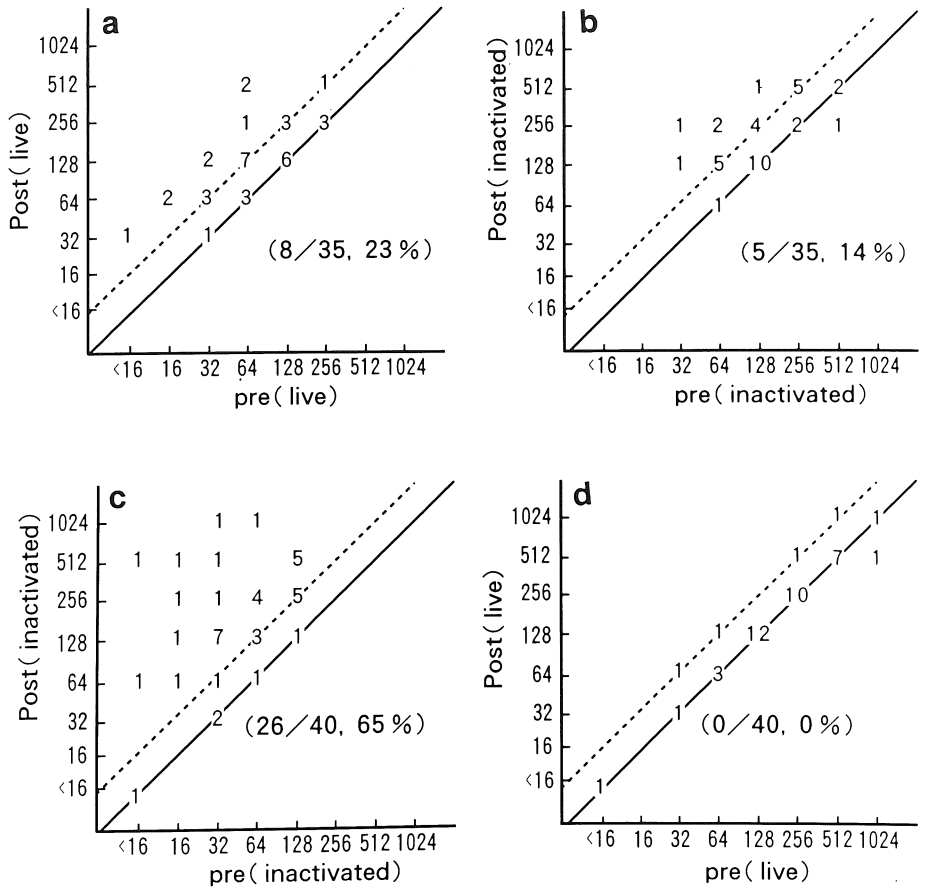

Figure 3 Serum HI antibody to A/Beijing H3N2. The interval between the two types of vaccines was 6 weeks. The abscissae give the reciprocals of prevaccination HI levels. The ordinates give the reciprocals of postvaccination HI levels. The serum samples were taken 6 weeks after vaccination. Numbers shown in the figure are the numbers of volunteers who showed corresponding pre- and postvaccination levels. Values in parentheses give the rates and percentages of volunteers who manifested fourfold or greater increases in serum HI levels after administration of the vaccine. (a) (b) HI levels of the volunteers who received live vaccine first and inactivated vaccine second. (a) Responses to live vaccine; (b) responses to inactivated vaccine. (c) (d) HI levels of the volunteers who received inactivated vaccine first and live vaccine second. (c) Responses to inactivated vaccine; (d) responses to live vaccine

serum HI levels after administration of inactivated vaccine. The remaining 11 had preimmunization serum HI levels $\geqslant 128$ and five of these 11 (45%) showed significant increases in serum HI levels after administration of inactivated vaccine. Those results indicated that: (1) inactivated vaccine stimulated systemic immune responses more strongly than live vaccine (*Figure 3c* 65% versus *Figure 3a* 23%, $p=0.0006$); (2) inactivated vaccine manifested a strong booster effect with regards to systemic immune responses, while live vaccine did not (*Figure 3b* 14% versus *Figure 3d* 0%, $p=0.0443$); (3) the results in *Figure 3a* showing the rate of fourfold or greater increases in serum HI levels (23%) in contrast to those of *Figure 3d* (0%), indicated that the previous administration of inactivated vaccine was effective in prevention of infection with live vaccine virus; and (4) inactivated vaccine induced systemic immune responses in volunteers with both low (serum HI levels of $\leqslant 64$) and high (serum HI levels of $\geqslant 128$) preimmunization serum antibody levels (*Figure 3c*), while live influenza virus vaccines induced immune responses only in volunteers with low preimmunization serum antibody levels (*Figure 3a*).

Figures 4 and *5* are serum HI antibody responses to B/Bangkok and A/Yamagata H1N1, respectively. Rates of fourfold or greater increases in serum HI levels after administration of inactivated vaccine and of live vaccine were 75% (*Figure 4c*) versus 57% (*Figure 4a*) for B/Bangkok ($p=0.1641$) and 30% (*Figure 5c*) versus 9% (*Figure 5a*) for A/Yamagata H1N1 ($p=0.0428$), respectively. Thus, inactivated vaccine stimulated systemic immune responses more strongly than live vaccine. Rates of fourfold or greater increases in serum HI levels after booster administration of inactivated vaccine and of live vaccine were 14% (*Figure 4b*) versus 5% (*Figure 4d*) for B/Bangkok ($p=0.3264$) and 17% (*Figure 5b*) versus 2% (*Figure 5d*) for A/Yamagata H1N1 ($p=0.0755$), respectively. Thus, inactivated influenza vaccine manifested stronger booster effects in systemic immune responses than live vaccine. General tendencies of the serum HI antibody responses observed with B/Bangkok and A/Yamagata H1N1 were the same as those observed with A/Beijing H3N2.

Figure 6 shows the mucosal specific IgA values to A/Beijing H3N2. The figures are arranged in the same way as those of serum HI antibody responses. Higher mucosal immune responses were obtained with inactivated vaccine than with live vaccine (*Figure 6c* 53% versus *Figure 6a* 17%, $p=0.0032$). Higher booster mucosal immune responses were obtained with inactivated vaccine than with live vaccine (*Figure 6b* 34% versus *Figure 6d* 10%, $p=0.0226$). The rate of fourfold or greater increases in mucosal specific IgA values of *Figure 6b* (17%) in contrast to that of *Figure 6d* (10%) indicated that mucosal immune response to live vaccine was suppressed in volunteers who had received inactivated vaccine previously. This means that proliferation of live vaccine virus over the upper respiratory mucosal surfaces was inhibited by prior local administration of inactivated vaccine. *Figures 7* and *8* show mucosal immune responses to B/Bangkok and A/Yamagata H1N1. Rates of fourfold or greater increases in mucosal specific IgA values after administration of inactivated or live vaccines were 43% (*Figure 7c*) versus 37% (*Figure 7a*) for B/Bangkok

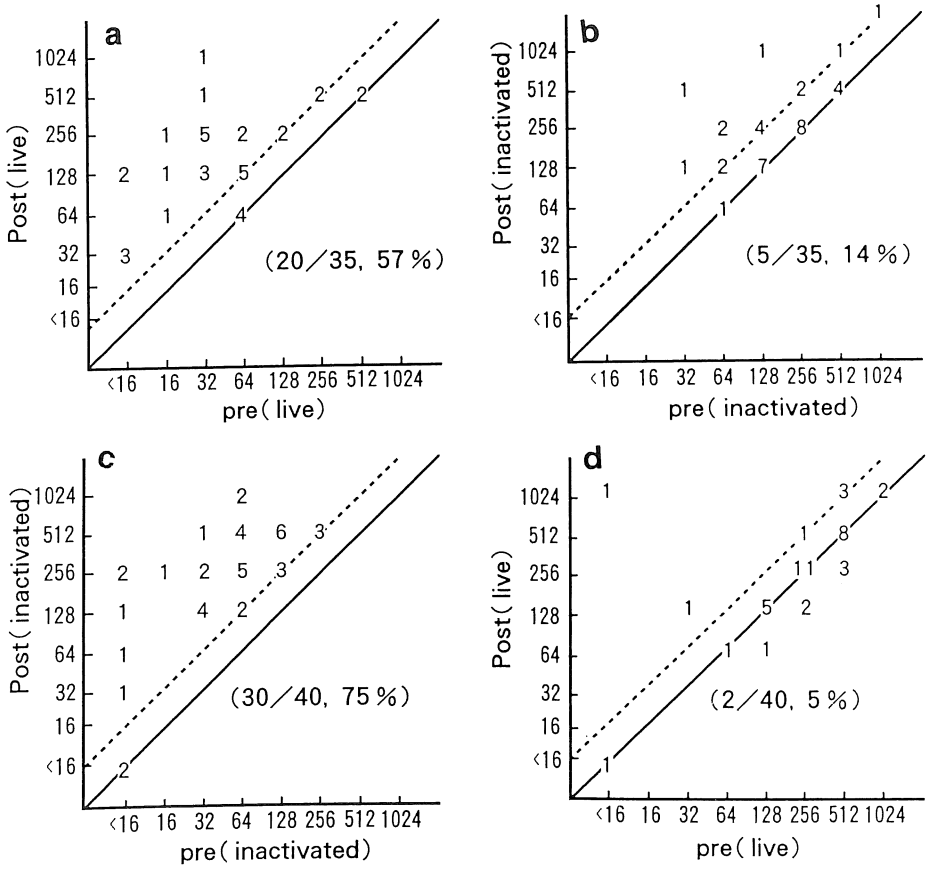

Figure 4 Serum HI antibody levels to B/Bangkok before and after administration of vaccine. For explanation, see legend to *Figure 3*

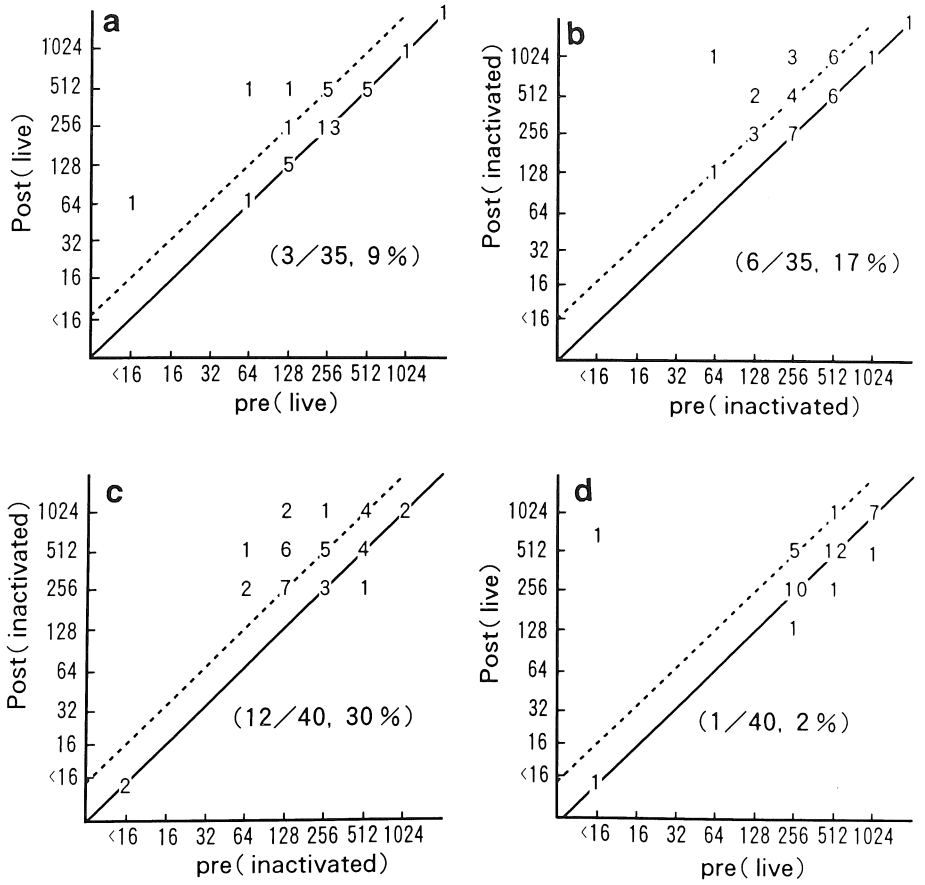

Figure 5 Serum HI antibody levels to A/Yamagata H1N1 before and after administration of vaccine. For explanation, see legend to *Figure 3*

($p=0.8132$) and 48% (*Figure 8c*) versus 29% (*Figure 8a*) for A/Yamagata H1N1 ($p=0.1493$), respectively. Rates of fourfold or greater increases in mucosal specific IgA values after booster doses of inactivated or live vaccines were 40% (*Figure 7b*) versus 10% (*Figure 7d*) for B/Bangkok ($p=0.0057$) and 46% (*Figure 8b*) versus 10% (*Figure 8d*) for A/Yamagata H1N1 ($p=0.0012$), respectively. Thus, inactivated influenza virus vaccine manifested stronger booster effects in mucosal immune responses than live vaccine. The general tendencies of the mucosal immune responses observed with B/Bangkok and A/Yamagata H1N1 were essentially the same as those observed with A/Beijing H3N2.

Direct challenge with live vaccine virus resulted in establishment of infection, determined by fourfold or greater increases in serum HI levels, in 23% for A/Beijing H3N2 (*Figure 3a*), 57% for B/Bangkok (*Figure 4a*) and 9% for A/Yamagata H1N1 (*Figure 5a*). Preimmunization with aerosol inactivated vaccine inhibited infection with live vaccine virus resulting in the decreases in establishment of infection to 0% for A/Beijing H3N2 (*Figure 3d*), 5% for B/Bangkok (*Figure 4d*) and 2% for A/Yamagata H1N1 (*Figure 5d*), respectively. Preimmunization with aerosol inactivated influenza vaccine reduced rates of fourfold or greater increase in mucosal specific IgA values from 17% (*Figure 6a*) to 10% (*Figure 6d*), from 37% (*Figure 7a*) to 10% (*Figure 7d*) and from 29% (*Figure 8a*) to 10% (*Figure 8d*) for A/Beijing H3N2, B/Bangkok and A/Yamagata H1N1, respectively.

DISCUSSION

Because of the lack of a sensitive and reliable method of evaluation, intranasally administered inactivated influenza virus vaccine has not been seriously considered as a vaccine for practical use. However, we have developed a very sensitive and reliable enzyme-linked immunoassay method which is critical for evaluation of antibody responses to mucosally administered vaccines. Using this technique we demonstrated specific IgA responses of the mucous membrane to inactivated influenza virus vaccines given intranasally. We have also demonstrated that this route of administration of inactivated influenza virus vaccine stimulated systemic immune responses[9].

The study reported in this paper was based on the observations described above that topically administered influenza virus vaccine stimulated both mucosal and systemic immune responses. One of the purposes of this study was to determine immunogenicity and efficacy of vaccines in a natural population. Therefore, no selection was undertaken in terms of preimmunization antibody levels and the two groups in the crossover test contained volunteers with various levels of preimmunization antibody. Our study revealed the important difference between live and inactivated influenza virus vaccines given intranasally. Live vaccine induced mucosal and systemic immune responses only in non-immune subjects while inactivated vaccine induced mucosal and systemic immune responses in both the non-immune and immune

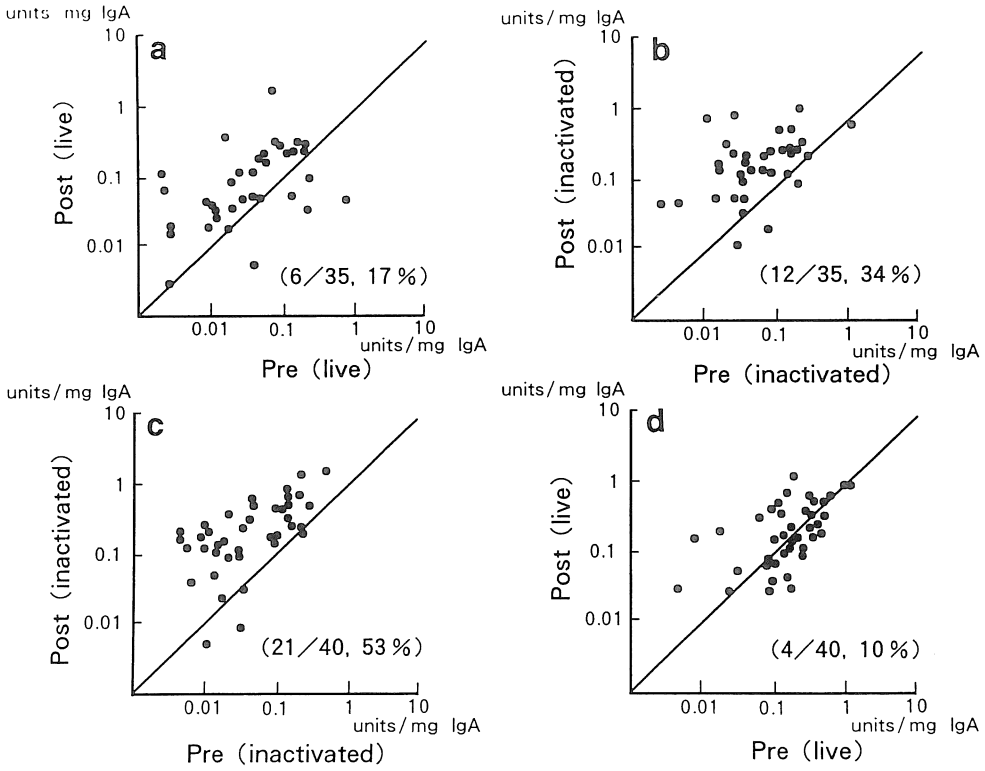

Figure 6 Values of specific IgA to A/Beijing H3N2 in nasal swab solution. The interval between the two types of vaccines was 6 weeks. The abscissae give the prevaccination specific IgA values. The ordinates give the postvaccination specific IgA values. Nasal swab samples were taken 6 weeks after vaccination. The values are expressed in terms of units mg^{-1} total IgA. Each point represents the result of one volunteer. The values in parentheses are the rates and percentages of volunteers who manifested fourfold or greater increases in mucosal specific IgA values after administration of the vaccine. (a) (b) Mucosal specific IgA responses of volunteers who received live vaccine first and inactivated vaccine second. (a) Mucosal specific IgA responses to live vaccine; (b) responses to inactivated vaccine. (c) (d) Mucosal specific IgA levels of volunteers who received inactivated vaccine first and live vaccine second. (c) Mucosal specific IgA responses to inactivated vaccine; (d) responses to live vaccine

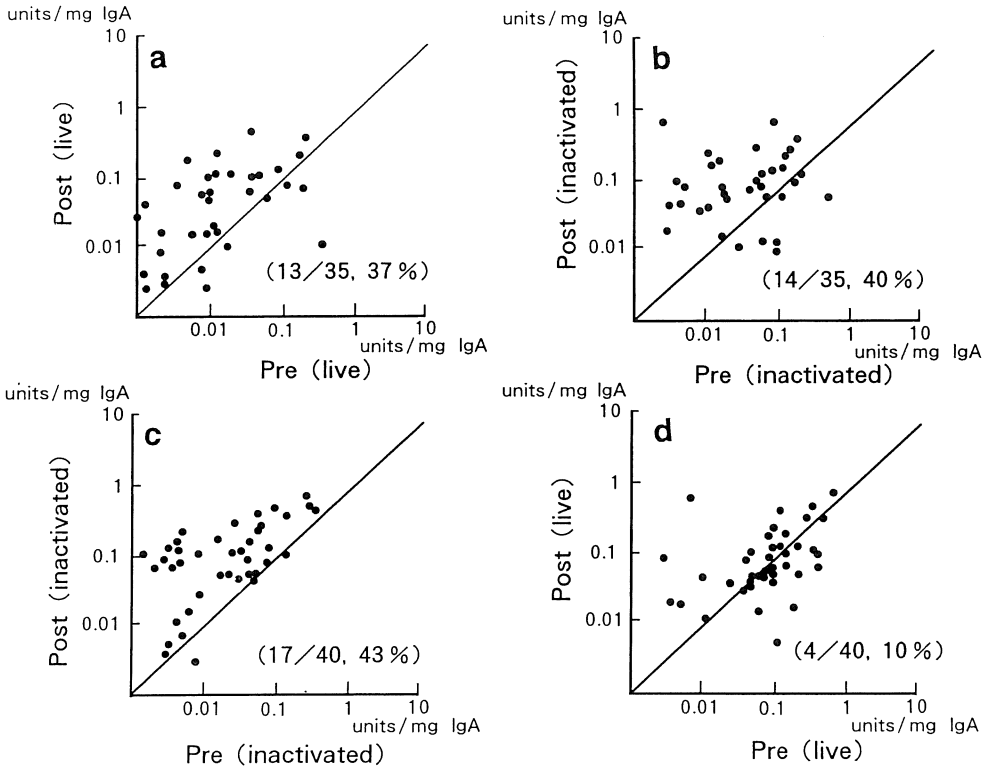

Figure 7 Mucosal IgA values specific to B/Bangkok. For explanation, see legend to *Figure 6*

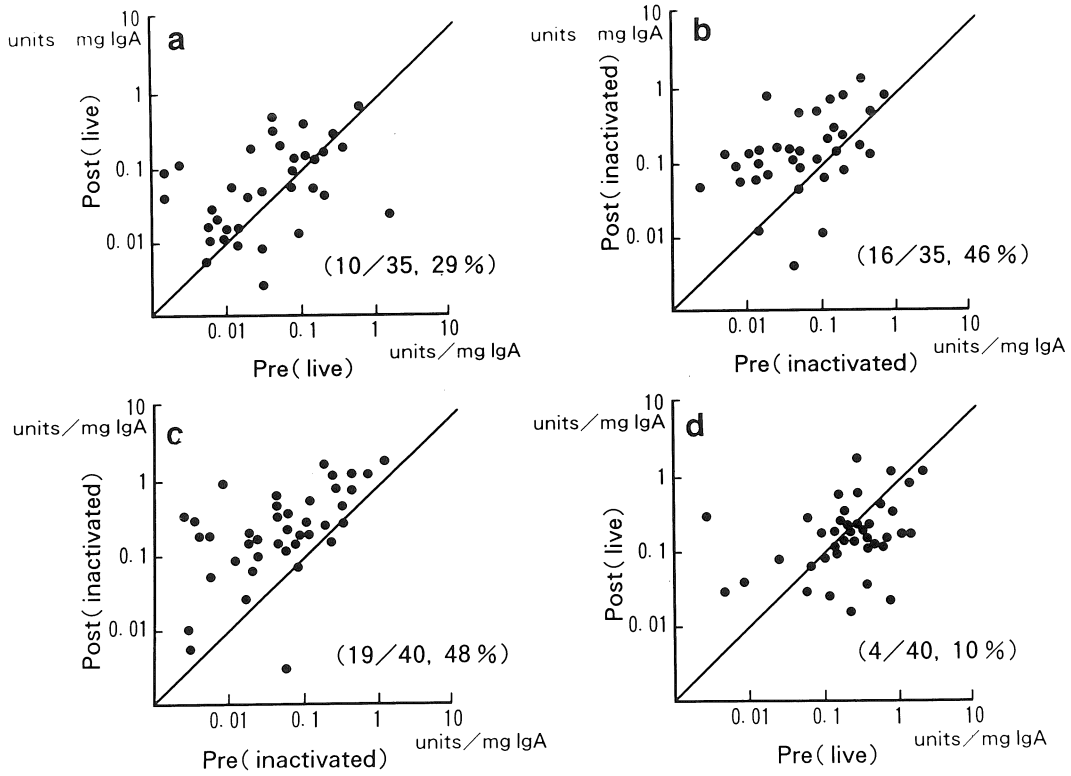

Figure 8 Mucosal IgA values specific to A/Yamagata H1N1. For explanation, see legend to *Figure 6*

volunteers. Evaluation of efficacy of the vaccine is important. To determine efficacy of mucosally administered inactivated influenza virus vaccines, a challenge test using cold-adapted reassortant live influenza vaccines as the challenge virus was designed. This is the first report on the efficacy of topically administered inactivated influenza virus vaccine by a challenge test.

It is now time to change the concept of control of influenza epidemics by the use of vaccines. Three types of influenza viruses infect humans, type A H1N1, type A H3N2 and type B. Mutations of influenza viruses are extremely common and antigenicity of the virus of a specified type never stays the same. In recent years two or three types of influenza virus prevailed every year and probably in almost every part of the world. Therefore, most people have already obtained some degree of immunity to all of these three types of influenza viruses. Each person has the opportunity to contract influenza but on the basis of varied degrees of immunity depending on previous exposures to influenza viruses. In consequence, when we plan to prevent epidemics of influenza, the strategy has to be the one that corresponds to this recent situation. In other words the vaccine should work effectively in those who are already partly immune to the type of vaccine virus and therefore it should be effective as a booster. Our study showed that topically administered inactivated influenza virus vaccine met this requirement. A study in unprimed mice suggested that locally administered inactivated influenza virus vaccine along with cholera toxin B subunit induced mucosal immune responses[11]. However, this adjuvant was not needed for a booster dose. Since mucosal influenza virus vaccines are going to be used in children and adults who have achieved some degree of immunity due to previous natural infections, it is not yet known whether the adjuvant enhances immune responses or not. Since safety of the adjuvant in humans has not been fully evaluated, especially when the vaccines are going to be used every year in the same individuals, further studies are warranted before mucosal inactivated influenza virus vaccine with any adjuvant is widely used.

CONCLUSION

Influenza, an air-borne disease, is a typical surface infection of the upper respiratory tract. To prevent proliferation of influenza virus on the respiratory mucosal surfaces, a vaccine which stimulates the common mucosal immune system is needed. To stimulate the common mucosal immune system a vaccine should be administered directly to mucosal surfaces.

Influenza is such a common disease that most people have some degree of immunity to influenza virus. Therefore a mucosal influenza virus vaccine which can enhance immunity of partially immune people as well as endorse immunity in non-immune people may be preferable.

Aerosol inactivated influenza virus vaccines manifested higher potency in raising the level of the mucosal and systemic immune status of the vaccinees than live influenza virus vaccine did. We conclude that more attention should be paid to aerosol inactivated influenza virus vaccines.

ACKNOWLEDGEMENTS

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