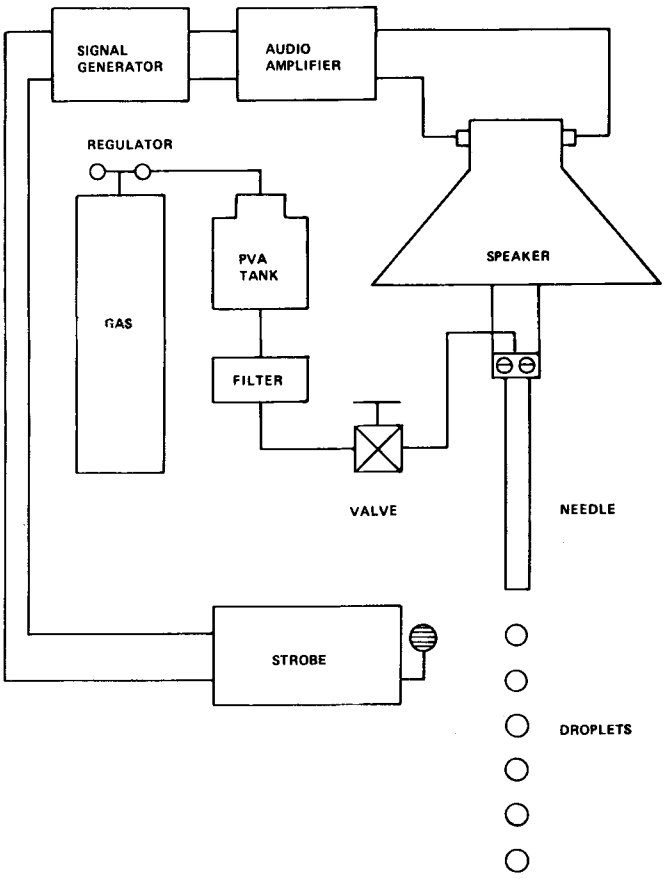

Figure 2—*Schematic diagram of droplet generator. [PVA = poly(vinyl alcohol).]*

provements may be achieved by modifying the polymerization kinetics.

Further polymeric shell research will be aimed at improving sphericity and increasing sizes. To this end, a device known as an acoustically modulated fluid jet has been designed and built (Fig. 2.) It is capable of producing uniformly sized droplets of polymer solution or melt. The solution to the problem of producing large (5 mm), hollow, spherical polymeric pellets will likely be solved through an understanding of uniform droplet production.

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Long-Term Studies of Microencapsulated and Adsorbed Influenza Vaccine Nanoparticles

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Received August 2, 1979, from the **Department of Pharmaceutical Technology, Swiss Federal Institute of Technology, Clausiusstr. 25, CH-8092 Zürich, Switzerland*, and the ‡*Sandoz Forschungsinstitut Gesellschaft M. B. H., Vienna, Austria.* Accepted for publication March 13, 1980.

Abstract □ Incorporation of antigens into nanometer-sized polymer particles was recently shown to lead to a good adjuvant effect. An optimal antibody response with killed influenza virus antigens was achieved with 0.5% poly(methyl methacrylate). Long-term experiments showed prolonged antibody response of polymer adjuvants with incorporated or adsorbed influenza virus. Adsorption also yielded an optimal adjuvant effect with 0.5% poly(methyl methacrylate). The antibody response was accompanied by protection of the mice against infection with mice-adapted influenza virus. In addition, the polymer vaccines were more stable against temperature inactivation than were vaccines with aluminum hydroxide or without adjuvants.

Keyphrases □ Vaccine, influenza—virus antigen incorporated into and adsorbed onto polymer nanoparticles, vaccines with and without adjuvant compared for protection in mice and against heat inactivation □ Polymers—poly(methyl methacrylate) and aluminum hydroxide used as adjuvants in influenza vaccine, effect of polymer on protection against influenza in mice and heat inactivation □ Microencapsulation—symposium, incorporation of influenza virus antigens into polymer nanoparticles compared with their adsorption onto polymer nanoparticles, vaccines with and without adjuvant evaluated for protection against influenza in mice and heat inactivation

Nanocapsules or nanoparticles, first described a few years ago (1–4), are nanometer-sized delivery systems for biologically active materials. This biologically active material may be totally or partially encapsulated, or it may be attached to these particles by adsorption or through chemical bonding.

At present, there are three distinctly different methods for nanoparticle production (1). The first method is micelle polymerization (2). In this process, polymerization is carried out in micelles or micelle-like structures in which the biologically active material and the polymerizable material are present.

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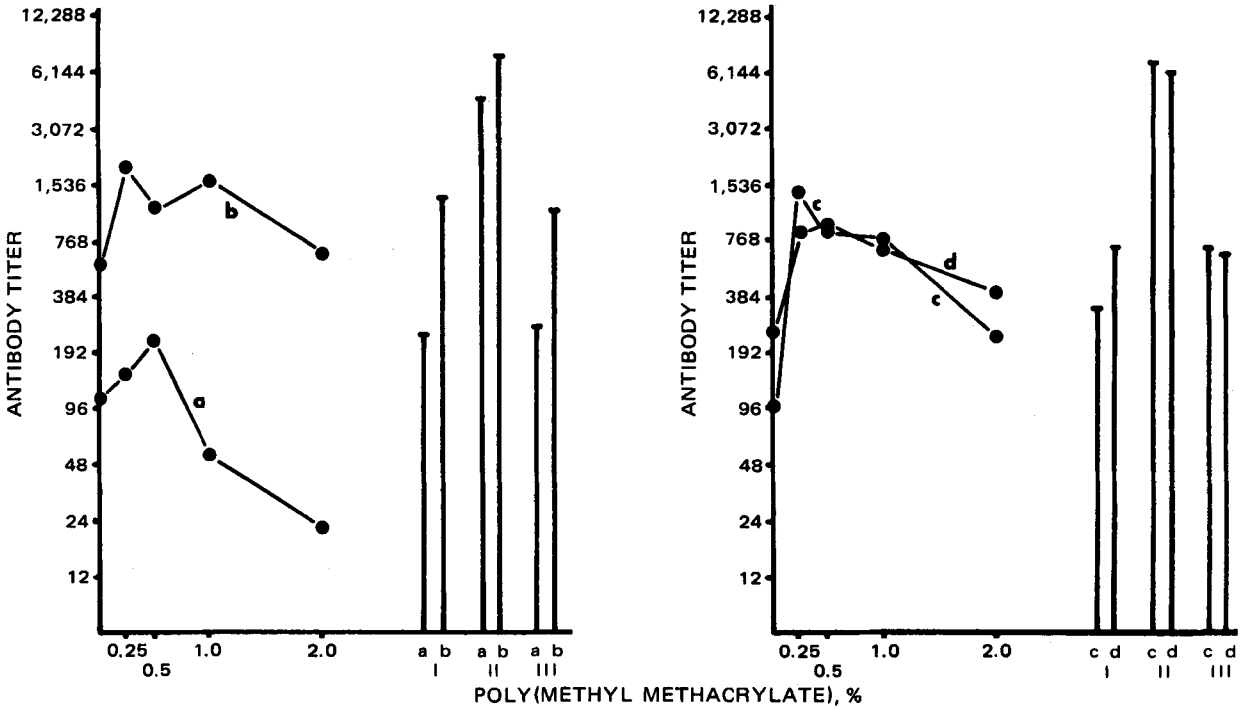

Figure 1—*Influence of increasing amounts of poly(methyl methacrylate) after antigen incorporation into the polymer on the antibody response after 4 (a), 8 (b), 12 (c), and 20 (d) weeks in guinea pigs. Boosting occurred after 4 weeks with fluid vaccines. The following adjuvants were used in addition: I, 0.1% aluminum hydroxide; II, Freund's complete adjuvant; and III, 0.5% methyl methacrylate + 0.5% acrylamide copolymer. The antigen was 400 IU of B/Hongkong 8/73 whole virions. For variabilities, see Table I. (Reproduced, with permission, from Ref. 3.)*

The second process involves polymerization of an aqueous solution of a polymerizable monomer (3, 5). During polymerization, the biologically active material is incorporated into the polymer particles due to interactions with the monomer or the polymer prior to or during polymerization. These interactions were demonstrated by the fact that 30% more monomer was clearly soluble in a virus suspension containing 3600 hemagglutinating units/ml than in a corresponding phosphate-buffered saline without virus (6). These 3600 hemagglutinating units correspond to 700 IU (international units), which approximates the content of various commercial vaccines.

The third process utilizes the rolling-up phenomenon of macromolecules (7). This conformational change occurs during the addition of a desolvating agent to a diluted solution of macromolecules. The rolled-up macromolecules entrap the biologically active material and may be hardened (for instance, with aldehydes) if gelatin is used as the macromolecule.

This report focuses on the second process, which is useful for vaccination when poly(methyl methacrylate) is used as the polymer (3).

EXPERIMENTAL

Virus—A_2/Hongkong/1/68 influenza virus (X-31 strain) was grown in 11-day-old fertile chicken eggs as described by Hoyle (8). The virus was purified and concentrated by density zonal centrifugation followed by dialysis against pH 7.2 phosphate-buffered saline. The final suspension was inactivated with 0.1% formaldehyde. The hemagglutinin content (9) was determined as described by Wood *et al.* (10).

The challenge virus, A_2/Hongkong/1/68 influenza virus, was adapted to mice by multiple passages through mouse lungs.

Influenza Subunits—The subunits containing hemagglutinin and neuraminidase were prepared as described by Bachmayer and coworkers (11, 12).

Table I—Antibody Titers (HI Titers) and Corresponding Log_2 of Guinea Pig Serum

Adjuvant	Parameter	Weeks 4	8	12	20
0.25% I	HI titer	146	1871	1360	838
	$\log_2 \bar{x} \pm SD$	7.19 ± 0.84	10.87 ± 1.15	10.41 ± 1.32	9.71 ± 0.99
0.5% I	HI titer	218	1136	843	891
	$\log_2 \bar{x} \pm SD$	7.77 ± 0.98	10.51 ± 1.09	9.72 ± 1.17	9.80 ± 0.99
1.0% I	HI titer	52.3	1607	768	709
	$\log_2 \bar{x} \pm SD$	5.71 ± 1.55	10.65 ± 0.82	9.58 ± 0.91	9.47 ± 0.78
2.0% I	HI titer	21.3	657	234	402
	$\log_2 \bar{x} \pm SD$	4.41 ± 1.51	9.36 ± 1.93	7.87 ± 1.58	8.65 ± 1.86
Fluid	HI titer	105	568	96	241
	$\log_2 \bar{x} \pm SD$	6.71 ± 1.63	9.15 ± 1.56	6.58 ± 1.63	7.91 ± 1.00
0.1% $Al(OH)_3$	HI titer	237	1287	340	709
	$\log_2 \bar{x} \pm SD$	7.89 ± 1.08	10.33 ± 0.87	8.41 ± 1.12	9.47 ± 1.05
Freund's adjuvant	HI titer	4420	7492	7042	6144
	$\log_2 \bar{x} \pm SD$	12.11 ± 0.62	12.87 ± 0.59	12.78 ± 0.66	12.58 ± 1.08
0.5% I + 0.5% polyacrylamide	HI titer	265	1105	704	644
	$\log_2 \bar{x} \pm SD$	8.05 ± 1.07	10.11 ± 0.94	9.46 ± 0.67	9.33 ± 1.26

[a] For vaccination scheme and specification of vaccines, see Fig. 1.

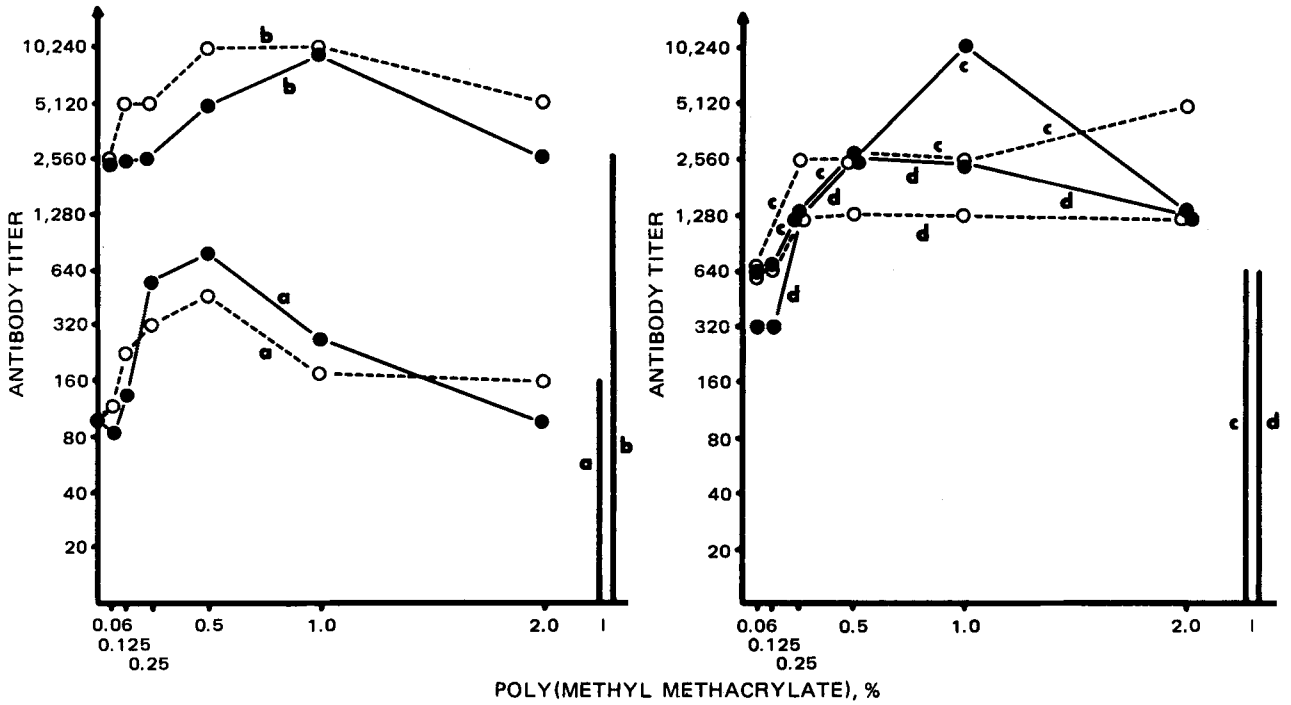

Figure 2—*Influence of different contents of poly(methyl methacrylate) on the antibody response of mice after 28 (a), 36 (b), 64 (c), and 92 (d) days. Boosting occurred after 28 days with the same vaccine as was used in the primary vaccination. Key: — ●, incorporation into poly(methyl methacrylate); - - - ○, adsorption onto poly(methyl methacrylate); and |, adsorption onto 0.2% aluminum hydroxide.*

Monomer—Methyl methacrylate[1] was purified from polymerization inhibitors by the methods described by Riddle (13) and Tessmar (14).

Incorporation of Antigen into Poly(methyl methacrylate) (I) Nanoparticles—Methyl methacrylate was dissolved in the antigen suspension and then polymerized with γ-rays (0.5 mrad) using a cobalt 60 source.

Adsorption of Antigen to I Nanoparticles—The monomer was polymerized as described; however, pH 7.2 phosphate-buffered saline was used instead of the antigen suspension. The resulting particles were centrifuged (680×*g* for 15 min) and resuspended in the antigen suspension after being washed three times with pH 7.2 phosphate-buffered saline.

Animals—Female NMRI mice[2], 20–24 g, were used.

Influence of I Content on Antibody Response—Whole influenza virions were used as the antigen. The vaccines had a hemagglutinin content of 630 IU/ml. Fourteen groups of 10 mice each received 0.1 ml/mouse, using one of the following adjuvant preparations: fluid without adjuvant; fluid incorporated into 0.06, 0.125, 0.25, 0.5, 1.0, and 2.0% I; fluid adsorbed onto 0.06, 0.125, 0.25, 0.5, 1.0, and 2.0% I; and fluid adsorbed onto 0.2% aluminum hydroxide.

The mice were boosted after 28 days with the same vaccine as was used in the primary vaccination. Blood was taken before boosting and after 36, 64, and 92 days.

Effect of Boosting on Antibody Response—Influenza subunits with a hemagglutinin content of 630 IU/ml were used as the antigen. Four groups of 20 mice each received 0.1 ml/mouse im. The following adjuvant preparations were used: fluid without adjuvant, fluid incorporated into 0.5% I, fluid adsorbed onto 0.5% I, and fluid adsorbed onto 0.2% aluminum hydroxide.

After 21 days, 10 mice of each group were boosted with the same vaccines as were used in the primary vaccination. Blood was taken after 21, 28, 42, 70, 125, and 180 days.

Antibody Determiation—The antibody determination was performed with the hemagglutination test, using the microtiter method (15, 16) in U plates with a 0.5% chicken erythrocyte suspension. Serum was pooled before determination.

Heat Stability—Whole influenza virions were used as the antigen. The vaccines had a hemagglutinin content of 21 IU/ml and were incorporated into 0.5% I, adsorbed onto 0.5% I, adsorbed onto 0.2% aluminum hydroxide, or without adjuvant. Each vaccine preparation was stored for 0, 15, 30, 60, 120, and 240 hr at 40°. Then 24 groups (four vaccines, six storage times) of 20 mice each received 0.1 ml/mouse of one vaccine preparation that had been stored for one of the specified time periods. One group of 20 mice served as a nonvaccinated control group. Twenty-eight days after immunization, the mice were challenged by spray infection with 50 times the LD_{50} value of the homologous mouse-adapted virus as described by Schulman and Kilbourne (17). The LD_{50} value of the challenge virus suspension was calculated according to Reed and Muench (18). Infection of the mice was recorded up to 9 days after the challenge, and the percentage of morbidity (lung lesions) was calculated.

RESULTS AND DISCUSSION

Influence of Poly(methyl methacrylate) (I) Content on Antibody Response—For vaccination purposes, it is desirable not to coat the viral antigen completely but to incorporate it partially into the particles so the antigen can react with the immunocompetent cells of the immunized host. The extent of coating can be monitored by the monomer content before polymerization (3). This effect is illustrated in Fig. 1.

Four weeks after vaccination of guinea pigs, the optimal antibody response was obtained with 0.5% I (3). After the 4-week blood samples were taken, all animals were boosted with a fluid vaccine. After 8 weeks and particularly after 12 and 20 weeks, the differences in antibody response between 0.5% vaccines and vaccines with a higher polymer content decreased more and more. The cause for this decrease could be polymer decomposition. Thus, more antigen would be exposed to the immunocompetent cells of the host and result in a prolonged immunostimulation. Indications of polymer decomposition were found recently[3]. Another reason for the decrease in differences between these vaccines could be that much less antigen is necessary to provoke a booster-type immune response compared with a primary vaccination.

Since the effectiveness of an immunization can depend on the animal species used, it was of interest to determine if the results obtained with guinea pigs could be reproduced in mice. Immunization of mice with influenza virus antigens seems to be relevant for adjuvant testing not only because the antibody response can be determined but because the protection after infection with live virus can be measured. Figure 2 shows the influence of different contents of I on the antibody response of mice. After 28 days, an optimum at 0.5% polymer was observed with influenza virions incorporated into nanoparticles and with virions adsorbed to

[1] Fluka AG, Buchs, Switzerland.
[2] Versuchstier-Zuchtanstalt Willy Gassner, Sulzfeld, Austria.
[3] Unpublished results.

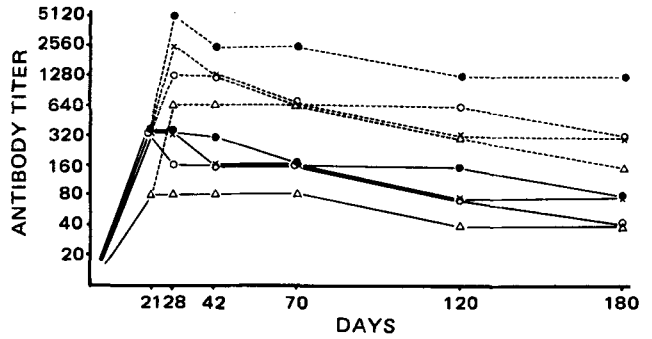

Figure 3—*Effect of boosting on the antibody response of mice. Key: —, unboosted groups; - - -, boosting after 21 days with the same vaccine as was used in the primary vaccination; ●, incorporation into 0.5% poly(methyl methacrylate); ○, adsorption onto 0.5% poly(methyl methacrylate); ×, adsorption onto 0.2% aluminum hydroxide; and △, fluid vaccine without adjuvant.*

nanoparticles. The optimum after incorporation shifted to 1% polymer after 36 and 64 days. The differences in antibody titers between the different polymer concentrations decreased after extended time with the incorporated and the adsorbed products. With the adsorbed products, the differences between the optimal and other polymer concentrations, however, were not as distinct as with the incorporated product. Both polymer vaccines were superior to aluminum hydroxide if optimal polymer concentrations were used.

It can be concluded that the antibody response in mice against influenza vaccines tested in these experiments is similar to the antibody response in guinea pigs. Moreover, when influenza is adsorbed onto nanoparticles, the antibody response is not as dependent on polymer content as it is after incorporation. However, incorporation into optimal polymer contents, in general, yielded higher antibody titers than adsorption. On the other hand, adsorption onto empty nanoparticles has the advantages that one batch of particles can be used with different antigens and that more drastic polymerization methods may be employed that otherwise would destroy the antigen.

Effect of I Adjuvants on Secondary Antibody Response—The effect of revaccination is shown in Fig. 3. Influenza incorporated into I always yielded the highest antibody response, whereas adsorption onto I and aluminum hydroxide yielded approximately the same response.

Mice Protection—Mice protection experiments are advantageous in that they can show the cooperation of all immune mechanisms following stimulation by vaccination and not only one immune mechanism, namely, antibody response. Although protection data obtained with mice

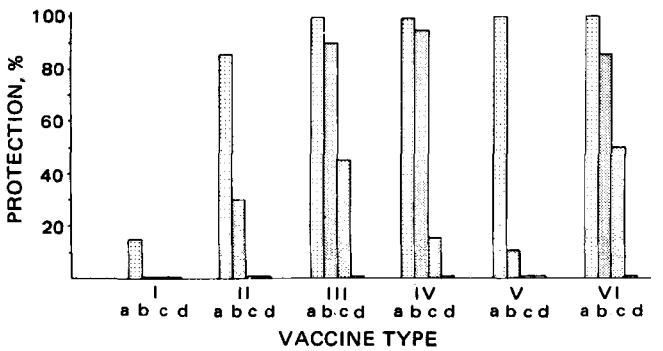

Figure 4—*Protection against mortality after immunization with A_2/Hongkong/1/68 (hemagglutinin content of the undiluted vaccine of 500 IU/ml) and challenge with 50 times the LD_{50} value of homologous mouse-adapted virus. The following vaccine preparations were used: Group I, incorporation of the undiluted vaccine into 0.5% poly(methyl methacrylate) and dilution together with the polymer; Group II, adsorption of the undiluted vaccine onto 0.5% poly(methyl methacrylate) and dilution together with the polymer; Group III, incorporation of the previously diluted vaccine into 0.5% poly(methyl methacrylate); Group IV, adsorption of the previously diluted vaccine onto 0.5% poly(methyl methacrylate); Group V, fluid vaccine without adjuvants; and Group VI, adsorption of the previously diluted vaccine onto 0.2% aluminum hydroxide. The vaccine dilutions were 1:50 (a), 1:500 (b), 1:5000 (c), and 1:50,000 (d). Levels of significance are shown in Table II.*

Table II—Levels of Significance of Protection against Mortality after Immunization with A_2/Hongkong/1/68 and Challenge with 50 Times the LD_{50} Value of Homologous Mouse-Adapted Virus Using the χ^2 Test of Snedecor and Cochran (20)

Vaccine[a]	Dilution	Statistical Significance of Protection
I	1:50	NS[b]
	1:500	NS
	1:5000	NS
	1:50,000	NS
II	1:50	$p < 0.001$
	1:500	$p < 0.05$
	1:5000	NS
	1:50,000	NS
III	1:50	$p < 0.001$
	1:500	$p < 0.001$
	1:5000	$p < 0.01$
	1:50,000	NS
IV	1:50	$p < 0.001$
	1:500	$p < 0.001$
	1:5000	$p < 0.01$
	1:50,000	NS
V	1:50	$p < 0.001$
	1:500	NS
	1:5000	NS
	1:50,000	NS
VI	1:50	$p < 0.001$
	1:500	$p < 0.001$
	1:5000	$p < 0.001$
	1:50,000	NS

[a] For vaccine number identification, see Fig. 4. [b] Not significant.

may not always be relevant for humans, they seem to correlate quite well with the effectiveness of the vaccines in humans; therefore, the mice protection model is widely used (17).

One disadvantage of mice protection experiments is that the mice cannot be vaccinated with the same antigen content contained in commercial vaccines since the protection is too good to differentiate between efficient and less efficient vaccines. For this reason, the vaccines have to be diluted.

Figure 4 shows the effect of dilution on six different vaccines. The experimental procedures were reported previously (19). The levels of significance (Table II) were calculated using the method of Snedecor and Cochran (20). The vaccines were diluted 1:50, 1:500, 1:5000, and 1:50,000. Group V represents the four dilutions of the fluid vaccine without adjuvants. In Group VI, 0.2% aluminum hydroxide was added to each dilution. In Group IV, 0.5% I nanoparticles were added by adsorption onto each

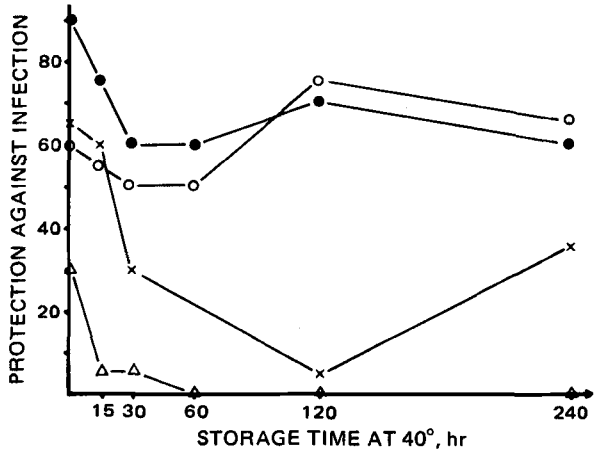

Figure 5—*Stability of vaccines against heat inactivation showing protection of mice against morbidity after immunization with vaccines that were stored at 40° for different time periods. The vaccines contained the following adjuvants: ●, incorporation into 0.5% poly(methyl methacrylate); ○, adsorption onto 0.5% poly(methyl methacrylate); ×, adsorption onto 0.2% aluminum hydroxide; and △, fluid vaccine without adjuvants. The mice were challenged with 50 times the LD_{50} value of homologous mice-adapted virus. Levels of significance are shown in Table III. The influence of heat inactivation tested by the 6 × 2 contingency table is shown in Table IV.*

Table III—Stability of Vaccines against Heat Inactivation: Levels of Significance of the Protection of Mice against Morbidity after Immunization with Vaccines that Were Stored at 40° for Different Times [a]

Vaccine	Storage Time at 40°, hr					
	0	15	30	60	120	240
0.5% Poly(methyl methacrylate) incorporation	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
0.5% Poly(methyl methacrylate) adsorption	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.01$	$p < 0.001$	$p < 0.001$
0.2% Aluminum hydroxide adsorption	$p < 0.001$	$p < 0.001$	$p < 0.05$	$p < 0.01$	NS[b]	$p < 0.05$
Fluid without adjuvant	$p < 0.05$	NS	NS	NS	NS	NS

[a] Levels of significance were determined using the χ^2 test. [b] Not significant.

Table IV—Effect of Heat Inactivation on the Stability of Vaccines Determined Using a 6 × 2 Contingency Table with χ^2 Test and Evaluation of Linear Regression [a] (21)

	Degrees of Freedom	0.5% Poly(methyl methacrylate) Incorporation, χ^2	0.5% Poly(methyl methacrylate) Adsorption, χ^2	0.2% Aluminum Hydroxide Adsorption, χ^2	Fluid without Adjuvant, χ^2
Regression	1	4.77*	0.50 NS	10.13***	13.27***
Rest	4	2.93 NS	3.07 NS	10.67*	8.50 NS
Total	5	7.70 NS	3.57 NS	20.80***	21.77***

[a] * = $p < 0.05$, *** = $p < 0.001$, and NS = not significant.

dilution; in Group III, each dilution was incorporated into 0.5% I nanoparticles.

In contrast to the latter two groups, in Group II the concentrated, undiluted vaccine was adsorbed onto 0.5% nanoparticles; in Group I, the undiluted vaccine was incorporated into 0.5% nanoparticles. Both vaccines then were diluted 1:50, 1:500, 1:5000, and 1:50,000. By this procedure the polymer was diluted together with the vaccine, resulting in 50- to 50,000-fold lower polymer concentrations in the final vaccine than in Groups III and IV. The resulting protection after adsorption onto polymer in Group II was equivalent to that of the fluid vaccine (Group V), whereas incorporation into the nanoparticles in Group I yielded much lower protection. Possibly too much antigen was covered by the polymer so that little or no immune stimulation resulted. These low polymer concentrations yielded no adjuvant effect. In contrast, incorporation of the previously diluted vaccine into 0.5% polymer resulted in good protection, as can be seen in Group III.

This experiment demonstrated that mice protection experiments have to be designed carefully and that the correct dilution as well as the correct dilution procedure has to be selected.

Heat Stability—Figure 5 and Table III show the heat stability of the vaccines tested in mice protection experiments. The vaccines were kept at 40° for 0, 15, 30, 60, 120, and 240 hr. The vaccines with adjuvants were more immunogenic than the fluid vaccine; after heating, the I vaccines were better than the aluminum hydroxide vaccine. For this reason, the nanoparticle vaccines, especially the adsorbed vaccine that is totally stable against heat inactivation (Table IV), hold promise for use when cooling conditions are not optimal.

CONCLUSIONS

Adsorption and incorporation of influenza virus antigen into nanoparticles yielded very effective vaccines. Their effectiveness was especially pronounced in long-term experiments. This result could be demonstrated by measurement of antibodies in mice and guinea pigs. The effectiveness was clearly dependent on the polymer content, especially after incorporation. With an optimal polymer content of 0.5%, incorporation yielded vaccines that were superior to all other products tested with the exception of Freund's complete adjuvant, which, however, is too toxic for vaccination. In addition, the polymer vaccines were more stable against heat inactivation than aluminum hydroxide-adjuvanted and fluid vaccines.

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