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New Adjuvants on a Polymethylmethacrylate Base

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A new type of adjuvant using influenza virus as an antigen is presented. The new adjuvant was produced by polymerizing monomeric methylmethacrylate in the presence of the antigen. As a comparison, influenza virus was added to previously polymerized polymethylmethacrylate particles. In animal experiments, the antibody response in mice and guinea pigs was measured. After polymerization in the presence of the antigen, the adjuvant effect was dependent on the methylmethacrylate concentration used, reaching an optimal concentration at 0.5%. This adjuvant preparation was considerably more effective than simple addition of virus to comparable polymethacrylate preparations or to aluminum hydroxide. The latter two adjuvants were approximately equivalent in effectiveness.

At present, mineral aluminum compounds and water-in-oil emulsions are the most widely used adjuvants for vaccines. Especially the emulsion adjuvants lead to high antibody levels of long duration.

The frequent occurrence of severe inflammatory reactions (2–4, 8, 16, 17) is a disadvantage. Aluminum compounds are better tolerated, but their adjuvant effect is much less pronounced. The pharmacologists Raskovà and Masek (10), therefore, proposed the application of polymethacrylic compounds for use as adjuvants.

Such polymer-antigen conjugates have already been tested. Steele (13) examined the adjuvant effect of polystyrene-antigen conjugates. Torrigiani and Roitt (15) and Freeman (1) used 0.8 and 0.5-μm polymethylmethacrylate particles. The observed adjuvant effects were poor, although an enhancement in 19S antibody production could be obtained with the polymethacrylate particles.

The following study was undertaken in an effort to develop a more effective, yet nontoxic, adjuvant using a similar polymer.

MATERIALS AND METHODS

Antigens. Whole formalin-inactivated, zonal centrifugation-purified influenza virions of the A2/Aichi, A2/Hongkong X-31, and B/Hongkong 8/73 strains served as antigens.

Monomers. Methylmethacrylate (Fluka) and acrylamide (CIBA-Geigy) were used as monomers. The purification of methylmethacrylate from polymerization inhibitors was carried out by the method described by Riddle (11) or Tessmar (14).

Preparation of polymeric particle adjuvants. The polymeric particles used as adjuvants were produced by γ-ray-induced polymerization of the monomers in the presence or absence of the antigen.

Polymerization in presence of the antigen. A specified amount of methylmethacrylate, ranging from 0 to 2%, was dissolved into the virus suspension. In some cases 0.5% acrylamide was added. Nitrogen was bubbled through the solution for 3 to 5 min with an injection needle to reduce oxygen, which acts as a polymerization inhibitor. Polymerization was achieved by γ-radiation (0.46 Mrad) with a ^{60}Co source.

Polymerization in absence of the antigen. The monomers were polymerized as described above, with phosphate-buffered saline instead of the virus suspension. The resulting particles were centrifuged (1,000 rpm for 10 min) and resuspended in the virus suspension after having been washed three times in phosphate-buffered saline.

Scanning electron microscopy. Scanning electron microscopy was used for determining the form and size of the polymer particles. The samples were produced by polymerization of 0.5% methylmethacrylate in the presence and absence of the antigen (A2/Aichi, content 32,000 hemagglutinin units/ml). After washing the resulting particles three times with twice distilled water, they were applied to a glass slide. After air drying at room temperature and coating with gold, samples were examined in a Cambridge Stereoscan MARK 2A.

Immunization procedures. Four groups of 20 female NMRI mice, weighing 20 g, received 62.5 chicken cell agglutination units/ml (CCA) of influenza vaccine (A2/Hongkong X-31) intraperitoneally per mouse. The following adjuvants were used: group 1, 0.5% PMMA (polymetylmethacrylate) plus 0.5% PAA (polyacrylamide) polymerized in presence of the antigen; group 2, 0.5% PMMA plus 0.5% PAA polymerized in absence of the antigen; group 3, 0.2% $Al(OH)_3$; group 4, fluid vaccine without adjuvant. Blood was taken before vaccination and after 20, 30, 40, and 50 days. Sera were inactivated by periodate treatment and heating at 56 C for 30 min before titration.

In a second experiment the influence of raising amounts of polymer on the adjuvant effect was exam-

ined. Eight groups of 25 guinea pigs received 400 IU of influenza virions (B/Hongkong 8/73) subcutaneously per animal. The following adjuvants were used: group 1, fluid vaccine without adjuvant; group 2, 0.25% PMMA; group 3, 0.5% PMMA; group 4, 1.0% PMMA; group 5, 2.0% PMMA; group 6, 0.5% PMMA plus 0.5% PAA; group 7, 0.1% $Al(OH)_3$; group 8, complete Freund adjuvant. Groups 1 to 6 were polymerized in presence of the antigen.

Blood was taken before immunization and after 4, 8, 12, and 20 weeks. All animals received a booster injection of 400 IU of B/Hongkong 8/73, without adjuvant, subcutaneously after 4 weeks. The serum was inactivated with receptor-destroying enzyme and by heating at 56 C before titration.

A third experiment studied the effect of different amounts of antigen. Twelve groups of 15 guinea pigs and 12 groups of 10 mice of both sexes (weighing 18 to 20 g) received specific amounts of influenza virions (B/Hongkong 8/73) subcutaneously. The following amounts of antigen and adjuvants were used: group 1, 800 IU of 0.5% PMMA plus 0.5% PAA; group 2, 80 IU of 0.5% PMMA plus 0.5% PAA; group 3, 8 IU of 0.5% PMMA plus 0.5% PAA (group 1 to 3 were polymerized in the presence of the antigens); group 4, 800 IU of 0.8% PMMA; group 5, 80 IU of 0.8% PMMA; group 6, 8 IU of 0.8% PMMA (all polymerized in the absence of the antigens); group 7, 800 IU of 0.1% $Al(OH)_3$; group 8, 80 IU of 0.1% $Al(OH)_3$; group 9, 8 IU of 0.1% $Al(OH)_3$; group 10, 800 IU of fluid without adjuvant; group 11, 80 IU of fluid without adjuvant; group 12, 8 IU of fluid without adjuvant.

The guinea pigs were bled after 0, 2, 4, 6, and 8 weeks. A subcutaneous booster injection of the same amount of antigen as used in the primary vaccination was given without adjuvant to all animals. The mice were not boosted and all were bled after 3 weeks. All sera were inactivated by receptor-destroying enzyme treatment and heating at 56 C for 30 min before titration.

Antibody determination. The antibody determination was performed (Tables 1 and 2) with the hemagglutination inhibition test, using the microtiter method (7, 12) in "V" plates in the automatic pipetting machine (Autotiter III, manufactured by Canalco) with 0.5% chicken erythrocytes.

RESULTS

Scanning electron microscopy. The scanning electron microscopic pictures show the polymer products obtained after polymerization of the methacrylic monomers in the presence (Fig. 1) and in the absence (Fig. 2) of the antigens. The products were agglomerates of tiny roundish particles ranging in size between 50 and 300 nm. No differences in the morphology were seen between particles polymerized in the presence or absence of the virions. No virus structures could be observed in either case.

Antibody responses in mice and guinea pigs. The antibody titer of mice after a single application of 62.5 CCA of influenza virus, using four different adjuvant preparations, is shown in Fig. 3. The best adjuvant effect was obtained with the PMMA/PAA material polymerized in the presence of the antigen. The antibody titers of this material were eight times higher than those obtained after aqueous

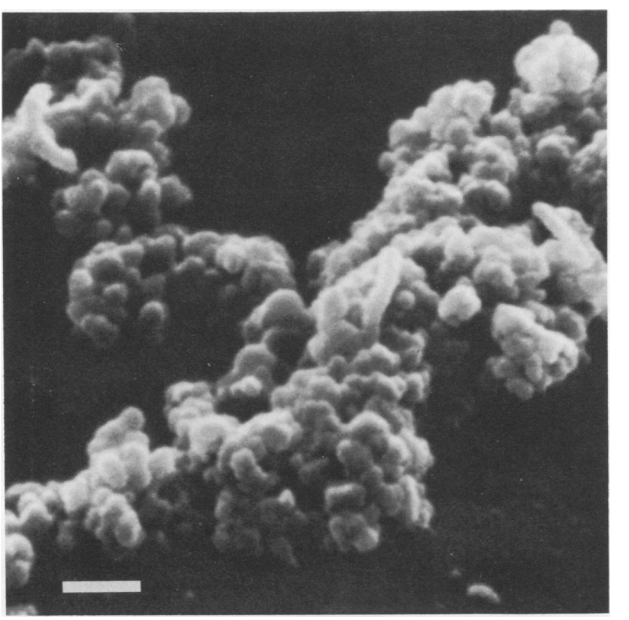

FIG. 1. *Agglomerates of polymer particles, produced by γ-ray-induced polymerization of 0.5% methylmethacrylate in the presence of influenza virions (32,000 hemagglutinin units/ml). No virion structures are visible. Marker, 500 nm.*

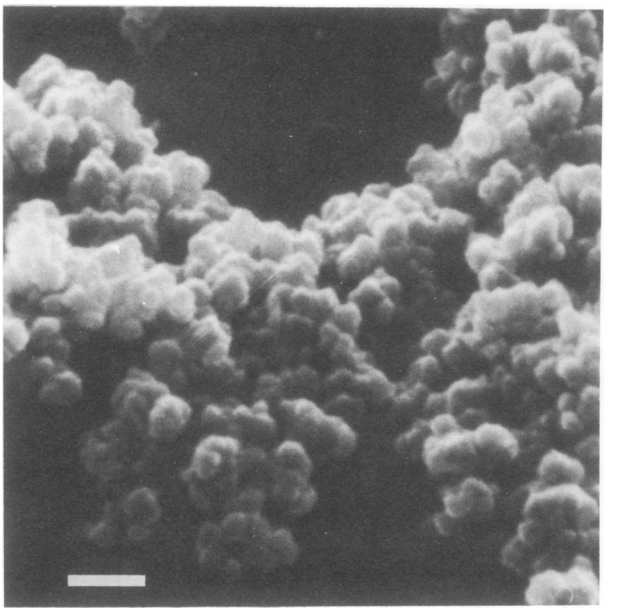

FIG. 2. *Agglomerates of polymer particles, produced by adding influenza virions (32,000 hemagglutinin units/ml) to a 0.5% PMMA suspension previously polymerized in the absence of antigen. No virus structures are visible. Marker, 500 nm.*

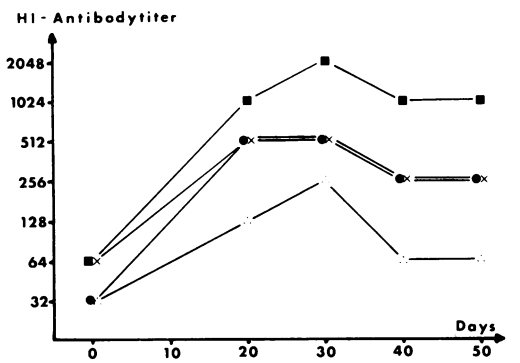

FIG. 3. *Influence of different adjuvants on the antibody response of mice. Adjuvants: ■, 0.5% PMMA plus 0.5% PAA polymerized in presence of the antigen; ●, 0.5% PMMA plus 0.5% PAA polymerized in absence of the antigen; ×, 0.2% Al(OH)$_3$; △, fluid vaccine without adjuvant. Antigen: 62.5 CCA of A2/Hongkong X-31, whole virions. Sera were pooled before titration. HI, Hemagglutination inhibition.*

vaccine. The antibody titers of the PMMA/PAA material, polymerized in the absence of the antigen, and the Al(OH)$_3$-adsorbed material were equivalent and were intermediate between the two other preparations.

Figure 4 demonstrates the effect of raising amounts of PMMA polymerized in the presence of the antigen. After 4 weeks, before boosting, the adjuvant effect reached an optimum with 0.5% PMMA. Increasing amounts of 1 to 2% PMMA depressed the antibody titers to values lower than those obtained with the aqueous vaccine. Titers approached values nearing 0 with 2% PMMA. After boosting with aqueous vaccine, the differences in the antibody titers between the PMMA preparations were less pronounced, and with increasing time the differences decreased. The titers of the preparation using 0.5% PMMA plus 0.5% PAA were very similar to the preparation using 0.5% PMMA alone. Freund complete adjuvant showed the best adjuvant effect during the whole observation period, whereas that of Al(OH)$_3$ was lower than that of the optimal PMMA concentration.

Figures 5 and 6 demonstrate the adjuvant effects of two different PMMA adjuvants compared to Al(OH)$_3$ and aqueous vaccines. In Fig. 5 guinea pigs served as experimental animals. The appearance of antibodies was delayed with 0.5% PMMA plus 0.5% PAA polymerized in the presence of the antigen: after 2 weeks, the antibody titers were lower than those of the other preparations. However, after boosting, this preparation appeared to have the best adjuvant effect. Furthermore, the decrease of the antibody titers was less pronounced for this preparation than for the other three. The PMMA-added preparation again reached intermediate titers, whereas in some cases (80 and 8 IU) the antibody titers of Al(OH)$_3$ were lower than those of the aqueous vaccine.

Figure 6 shows the antibody response in mice

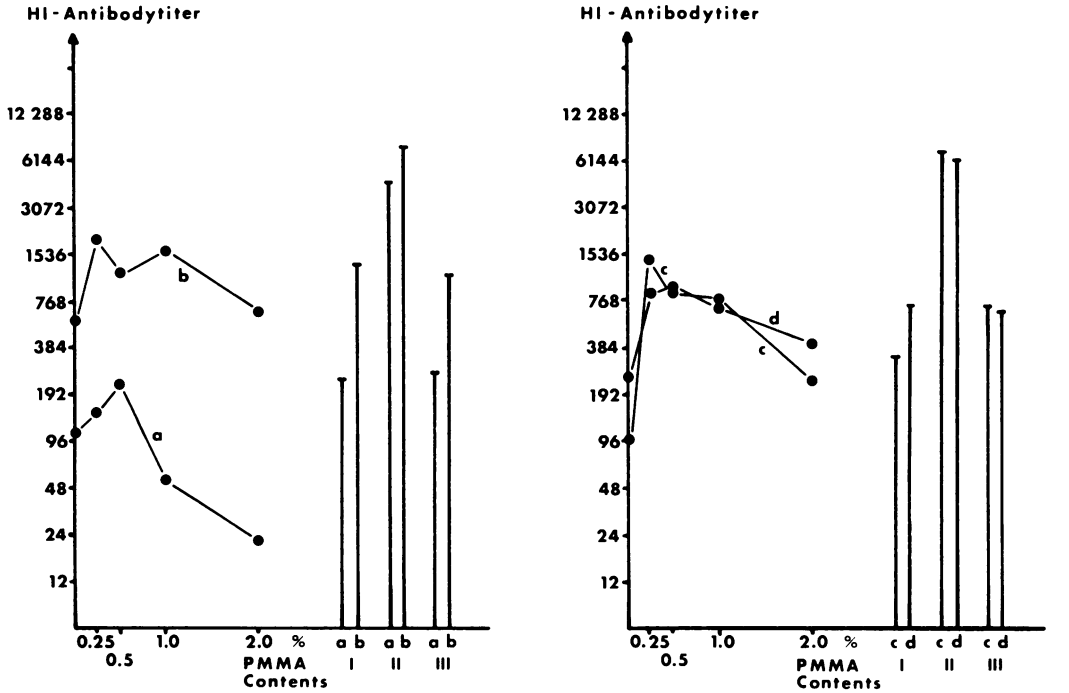

FIG. 4. *Influence of increasing amounts of PMMA, polymerized in presence of the antigen, on the adjuvant effect after (a) 4, (b) 8, (c) 12, and (d) 20 weeks in guinea pigs. Boosting after 4 weeks with fluid vaccines. The following adjuvants were used in addition: I, 0.1% Al(OH)$_3$; II, Freund complete adjuvant; III, 0.5% PMMA plus 0.5% PAA. Antigen: 400 IU of B/Hongkong 8/73, whole virions. Sera were titrated individually; variations see Table 1. HI, Hemagglutination inhibition.*

TABLE 1. *Antibody titers (hemagglutination inhibition titers) and corresponding log$_2$ with standard deviation of guinea pig sera*[a]

Adjuvants	Weeks			
	4	8	12	20
0.25% PMMA	146[b]	1871	1360	838
	7.19 ± 0.84[c]	10.87 ± 1.15	10.41 ± 1.32	9.71 ± 0.99
0.5% PMMA	218	1136	843	891
	7.77 ± 0.98	10.15 ± 1.09	9.72 ± 1.17	9.80 ± 0.99
1.0% PMMA	52.3	1607	768	709
	5.71 ± 1.55	10.65 ± 0.82	9.58 ± 0.91	9.47 ± 0.78
2.0% PMMA	21.3	657	234	402
	4.41 ± 1.51	9.36 ± 1.93	7.87 ± 1.58	8.65 ± 1.86
Fluid	105	568	96	241
	6.71 ± 1.63	9.15 ± 1.56	6.58 ± 1.63	7.91 ± 1.00
0.1% Al(OH)$_3$	237	1287	340	709
	7.89 ± 1.08	10.33 ± 0.87	8.41 ± 1.12	9.47 ± 1.05
Freund adjuvant	4420	7492	7042	6144
	12.11 ± 0.62	12.87 ± 0.59	12.78 ± 0.66	12.58 ± 1.08
0.5% PMMA plus	265	1105	704	644
0.5% PAA	8.05 ± 1.07	10.11 ± 0.94	9.46 ± 0.67	9.33 ± 1.26

[a] Vaccination scheme and specification of vaccines in Fig. 4.
[b] Hemagglutination inhibition titer.
[c] Log$_2$ ± standard deviation.

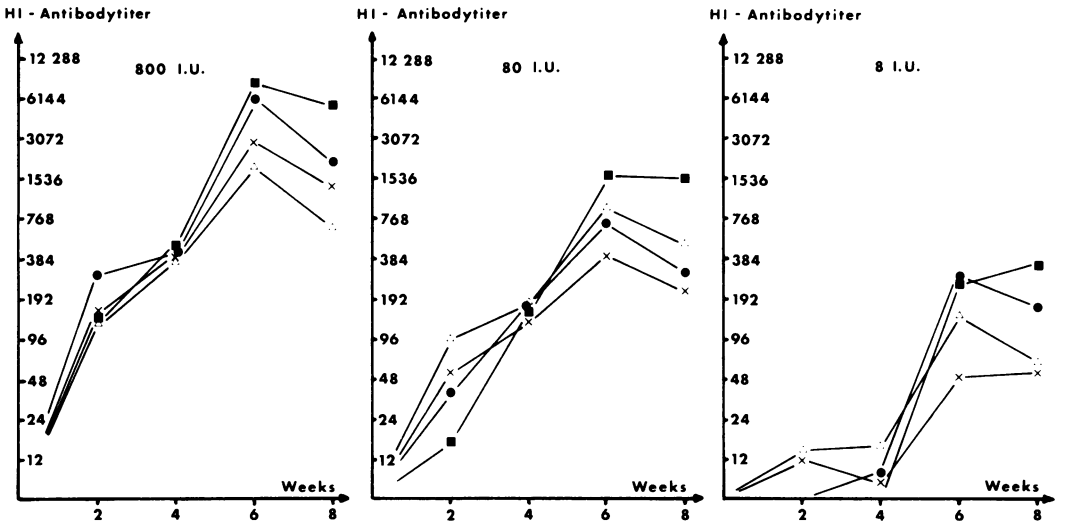

FIG. 5. *Antibody response after immunization of guinea pigs with B/Hongkong 8/73, using different adjuvants and antigen concentrations. Boosting after 4 weeks with fluid vaccines containing the same amount of antigen as in the primary vaccination. Adjuvants: ■, 0.5% PMMA plus 0.5% PAA polymerized in presence of the antigens; ●, 0.8% PMMA polymerized in absence of the antigens; ×, 0.1% Al(OH)$_3$; △, fluid vaccine. Sera were titrated individually; variations see Table 2. HI, Hemagglutination inhibition.*

TABLE 2. *Antibody titers (hemagglutination inhibition titers) and corresponding log$_2$ with standard deviation of guinea pig sera*[a]

Adjuvants	Antigen contents (IU)	Weeks			
		2	4	6	8
0.5% PMMA plus 0.5% PAA	800	143[b]	455	7996	5575
		7.16 ± 1.78[c]	8.83 ± 1.98	12.96 ± 0.52	12.44 ± 1.57
	80	16.5	159	1646	1536
		4.04 ± 0.88	7.31 ± 2.01	10.68 ± 0.89	10.58 ± 1.25
	8	<4	<4	259	341
		<2	<2	8.01 ± 0.79	8.41 ± 0.75
0.8% PMMA	800	281	441	6144	2027
		8.13 ± 1.21	8.78 ± 0.83	12.58 ± 0.71	10.98 ± 1.52
	80	38.1	165	707	297
		5.21 ± 1.63	7.36 ± 1.48	9.46 ± 1.36	8.21 ± 1.40
	8	<4	9.29	272	167
		<2	3.21 ± 1.41	8.08 ± 1.28	7.38 ± 1.10
0.1% Al(OH)$_3$	800	150	406	2886	1356
		7.22 ± 1.60	8.66 ± 1.66	11.49 ± 0.94	10.40 ± 1.08
	80	54.0	130	414	224
		5.75 ± 1.09	7.02 ± 0.88	8.69 ± 1.23	7.80 ± 0.97
	8	12.6	7.86	50.7	54.4
		3.65 ± 0.88	2.97 ± 0.77	5.66 ± 1.36	5.76 ± 1.07
Fluid	800	132	384	1891	669
		7.04 ± 1.51	8.58 ± 1.28	10.88 ± 1.57	9.38 ± 1.32
	80	101	167	946	507
		6.65 ± 1.38	7.38 ± 1.40	9.88 ± 1.16	8.98 ± 1.43
	8	14.0	15.4	146	65.1
		3.81 ± 1.09	3.94 ± 1.50	7.18 ± 1.51	6.02 ± 1.21

[a] Vaccination scheme and specification of vaccines in Fig. 5.
[b] Hemagglutination inhibition titer.
[c] Log_2 ± standard deviation.

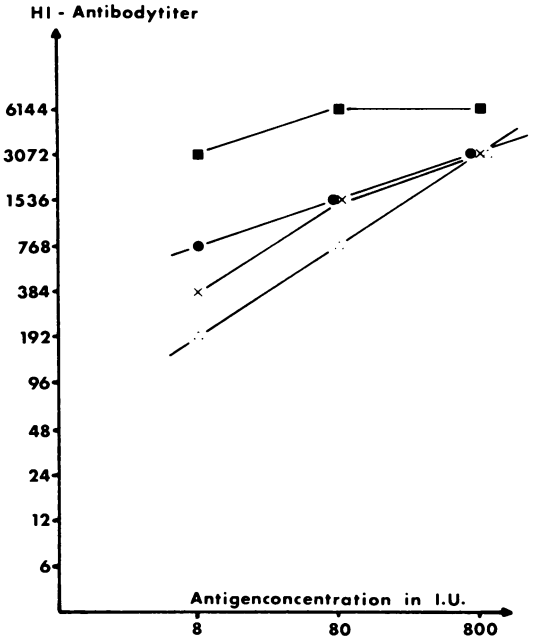

FIG. 6. *Dependence of the antibody response of mice on the antigen concentration after 3 weeks, using the adjuvants listed in Fig. 5. Antigen: B/Hongkong 8/73. Sera were pooled before titration. HI, Hemagglutination inhibition.*

after 3 weeks. The antibody titer was related to the amount of antigen used in all vaccines examined. PMMA (0.5%) plus PAA (0.5%) polymerized in the presence of the antigen again proved to be the best adjuvant, showing the best effect in vaccines with a low antigen content. PMMA (0.8%), polymerized in the absence of the antigen, and $Al(OH)_3$ show similar antibody titers. The titer of the aqueous vaccines was the lowest, showing a marked decrease in antibody response in comparison to the other preparations at the lower antigen doses.

DISCUSSION

These results clearly demonstrate that PMMA polymerized in the presence of the antigen, if used in optimal concentration, represents a potent new adjuvant in guinea pigs and mice. The effect was distinctly superior to that of $Al(OH)_3$ in all series of tests. However, the effectiveness of this product does not reach that of Freund complete adjuvant.

By polymerizating methylmethacrylate in the absence of the antigen and by adding influenza virions to the polymer product another adjuvant was obtained. Its effectiveness was not as good as that of the comparable PMMA product polymerized in the presence of the antigen. However, the adjuvant effect was as good or better (Fig. 5) than that of $Al(OH)_3$. This result contrasts with the findings of Torrigiani and Roitt (15), and to a lesser extent with those of Freeman (1), who found only poor adjuvant effects of PMMA particles. The reason for this could be differences in antigens and/or size and morphology of the particles. They used particles of 500 or 800 nm, (made by Bofors, Nobelkrut, Sweden) that have a round, rather smooth surface. The PMMA materials used here consist of agglomerates of particles with a highly structured surface. This highly structured surface probably has a strong adsorption capacity and could be responsible for the good adjuvant effect.

Unfortunately, the scanning electron microscopical method is insufficient for the exact determination of the morphological structures of the polymer products because of the 30-nm-thick gold layer due to the preparation method.

The scanning electron microscopical pictures show no differences in size or morphological structures of the products, polymerized either in the presence or absence of the antigen. However, there is a considerable difference in the antibody response between these preparations, though they contain the same amount of the plastic material (Fig. 3). This means that the influenza virions are not simply adsorbed to the PMMA particles after polymerization in the presence of the antigen. Rather, the virions undergo certain interactions with the methacrylate in the monomeric state or during polymerization. Virus-lipid interactions of influenza viruses were observed by Noll and Younger (6, 18). Due to such virus-lipid interactions, the virus could perhaps be coated by the developing polymer to a certain extent during the polymerization process.

This hypothesis could explain the 4-weeks result shown in Fig. 4. Increasing amounts of monomers could lead to a greater extent of coating the virion. This coating could be responsible for the adjuvant effect: less extensive coating leads to a good effect (PMMA contents around 0.5%), whereas more extensive coating deactivates so much antigen that the antibody titers decrease.

This coating hypothesis could also be an explanation for the time-delayed titer response appearing in Fig. 5: the coating of antigen surface could result in a depot effect, at first delaying and later stimulating contact with the immunocompetent cells.

The extent of coating seems to be mainly correlated to the amount of PMMA used. Even after the use of low amounts of antigen, where the ratio antigen/PMMA is low, a good adjuvant effect could be obtained. This could mean

that the virus-methacrylate interaction, taking place before or during polymerization, is an adsorption process whereby the methacrylate is adsorbed to the influenza virions. In the case of adsorption, the amount of methacrylate interacting with the virus should be dependent mainly upon the concentration of the monomer in the outer phase.

Acrylamide was used as an additive, to obtain a less hydrophobic product (polyacrylamide is water soluble). However, as Fig. 4 demonstrates, the addition of acrylamide has no influence on antibody response. Because of the severe toxic side effects of acrylamide, addition of this compound should be abandoned.

The antigen dose/antibody response relations were nearly linear for the same adjuvant preparations (Fig. 5 and 6), confirming the results of Mauler et al. (5). However, in contrast to their findings, curves did not run parallel. The new adjuvants seem to be especially effective with lower antigen concentrations. Therefore, perhaps this adjuvant could also be a good stimulant for poor immunogens. The method of polymerization in the presence of the antigen may not be applicable for all antigens, because certain methacrylate-virus interactions seem to have to precede the polymerization and coating procedure.

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