In Vitro Studies of Poly(methyl Methacrylate) Adjuvants

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Abstract □ Poly(methyl methacrylate) adjuvants, prepared by polymerizing monomeric methyl methacrylate in the presence of influenza virions or by addition of the virions to previously polymerized poly(methyl methacrylate) particles, were studied by means of the hemagglutination test, antibody binding, and electron microscopy. The results indicated that the virions were coated partly when the polymerization was carried out in the presence of the virus, whereas the virions were probably adsorbed when added to polymerized particles.

Keyphrases □ Poly(methyl methacrylate) adjuvants—for influenza vaccine, preparation, evaluation by hemagglutination, antibody binding, and electron microscopy □ Adjuvants, poly(methyl methacrylate)—for influenza vaccine, preparation, evaluation by hemagglutination, antibody binding, and electron microscopy □ Influenza vaccines—preparation of poly(methyl methacrylate) adjuvants, evaluation by hemagglutination, antibody binding, and electron microscopy □ Vaccines, influenza—preparation of poly(methyl methacrylate) adjuvants, evaluation by hemagglutination, antibody binding, and electron microscopy □ Virus vaccines, influenza—preparation of poly(methyl methacrylate) adjuvants, evaluation by hemagglutination, antibody binding, and electron microscopy

A previous publication (1) reported the preparation of potent new adjuvants for influenza vaccines. The new adjuvants were produced by γ-ray-induced polymerization of monomeric methyl methacrylate in the presence or absence of influenza virus. In the case of polymer preparation in the absence of the antigen, the virus was later added to the polymer particles. These two methods resulted in adjuvants characterized by different antibody-inducing properties.

The present investigation obtained additional information on the two different adjuvants.

EXPERIMENTAL

Virus—Whole formalin-inactivated, zonal centrifugation-purified influenza virions of the A2/Aichi and the A2/Hongkong X-31 strains were used.

Production of Clear Virion Suspension—The following simplified version of a purification method (2) was used. Allantoic fluid, containing 4800 hemagglutinating units/ml of A2/Hongkong X-31, was taken from embryonated chicken eggs that had been infected with live virus. Then 600 ml of a 1.5% chicken erythrocyte suspension was centrifuged at 1500 rpm for 15 min. The deposit was resuspended in 75 ml of the allantoic fluid by gentle agitation. This suspension was placed in an ice bath for 1–2 hr, the cells being kept in suspension by gentle agitation, and then the suspension was centrifuged again for 15 min at 1500 rpm.

The deposit (erthrocytes plus adsorbed virions) was washed two times by resuspending in 75 ml of phosphate-buffered saline at pH 7.2 and centrifuging under the described conditions. After washing, the deposit was resuspended in 40 ml of phosphate-buffered saline at pH 7.2; the virus was eluted by placing the suspension in a water bath at 37 ± 1° for 2 hr. The cells were then removed by centrifugation, and the supernatant fluid served as the clear virion suspension (content = 3200 hemagglutinating units/ml).

Production of Antibody Solution—Two guinea pigs were given 2 ml of an influenza vaccine (A2 / Hongkong X-31, 3200 hemagglutinating units/ml) containing 50% of complete Freund adjuvant. After 7 and 14 days, the animals were boosted with the same vaccine. Twenty-four days after the primary vaccination, blood was taken from the animals. The serum was inactivated by heating at 56° for 30 min and was then diluted with phosphate-buffered saline at pH 7.2 to obtain an antibody solution with a hemagglutination inhibition titer of 1600.

Table I—Hemagglutination Titers of Poly(methyl Methacrylate) Adjuvant Influenza Vaccines and Intermediate Products

Treatment	Hemagglutination Titer
Fluid virus suspension, untreated	3200
Fluid virus suspension, γ-radiated	3200
Fluid virus suspension + 0.8% monomeric methyl methacrylate, not radiated	3200
0.8% Poly(methyl methacrylate) particles without virions	160
Virus suspension + 0.8% poly(methyl methacrylate), polymerized in presence of virions	160
Virus suspension + 0.8% poly(methyl methacrylate), polymerized in absence of virions	1200

Monomer—Methyl methacrylate[1] was purified from polymerization inhibitors by the method described by Riddle (3) or Tessmar (4).

Preparation of Poly(methyl Methacrylate) Adjuvants—The poly(methyl methacrylate) adjuvants were produced by polymerization in the presence or absence of the virions (1).

Polymerization in Presence of Virions—An amount of methyl methacrylate, 0–2% (Tables I and II and Figs. 1 and 2), was dissolved

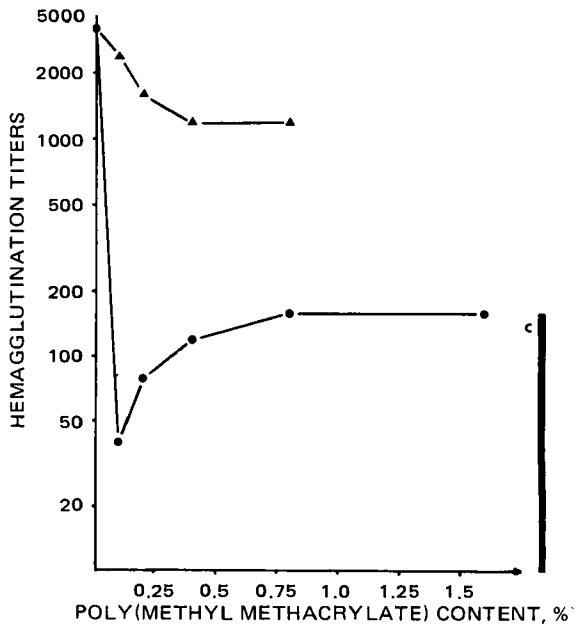

Figure 1—*Dependency of the hemagglutination titers on the poly(methyl methacrylate) content of influenza vaccines. Key: ●, polymerization in the presence of the virus; ▲, polymerization in the absence of the virus; and C, 0.8% poly(methyl methacrylate) particles without virus.*

[1] Fluka, Fluka AG, Buchs, Switzerland.

Table II—Hemagglutination Titers of Poly(methyl Methacrylate) Adjuvant Influenza Vaccines with Increasing Poly(methyl Methacrylate) Contents

Poly(methyl Methacrylate) Content, %	Mean ($\bar{x}$) and Standard Deviation (s)	Polymerization in Presence of Virus		Polymerization in Absence of Virus	
		Complete Vaccine	Supernatant Fluid	Complete Vaccine	Supernatant Fluid
0.0	$\bar{x}$	960	960	960	960
	s	109	109	109	109
0.025	$\bar{x}$	120	64	—	—
	s	13	0		
0.05	$\bar{x}$	48	1	320	12
	s	25	0	65.5	1.65
0.1	$\bar{x}$	20	0	240	2
	s	7		135.5	0
0.2	$\bar{x}$	60	0	160	1
	s	13		0	0
0.4	$\bar{x}$	120	0	160	0
	s	4.5		65.5	
0.6	$\bar{x}$	160	0	—	—
	s	26			
0.8	$\bar{x}$	160	0	320	0
	s	0		0	
1.0	$\bar{x}$	160	0	—	—
	s	26			
2.0	$\bar{x}$	240	0	—	—
	s	65.5			
1.0 Control[a]	$\bar{x}$	160	0	160	0
	s	0		0	
2.0 Control[a]	$\bar{x}$	240	0	240	0
	s	65.5		65.5	

[a] Poly(methyl methacrylate) particles without virus.

in the virus suspension. The oxygen content was reduced by bubbling nitrogen for 3–5 min through the suspension by means of an injection needle. The polymerization was carried out by γ-radiation with a dosage of 0.46 Mrad with a cobalt-60 source.

Polymerization in Absence of Virions—The monomer was polymerized as already described, using phosphate-buffered saline at pH 7.2 instead of the virus suspension. The resulting particles were centrifuged (1000 rpm for 10 min) and resuspended in the virus suspension after being washed three times in phosphate-buffered saline at pH 7.2.

Monomer Solubility—Erlenmeyer flasks (150 ml) were filled with 100.0 ml each of one of the following fluids: double-distilled water, phosphate-buffered saline at pH 7.2, or clear virion suspension. The fluids were placed in a water bath at 20.0 ± 0.1° and stirred with a magnetic stirrer. Methyl methacrylate was added with a 0.05-ml repeating pipet[2] and, in a repetition of the experiment drop by drop, with a 0.01-ml graded microburet. The clearly soluble amount of methyl methacrylate was measured (Table III).

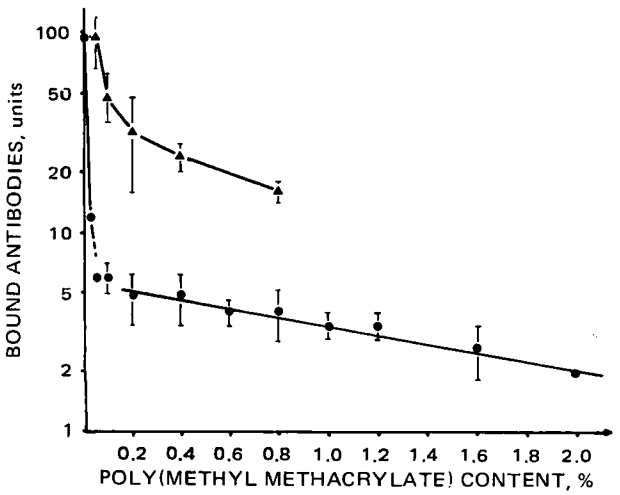

Figure 2—*Dependency of the amount of bound antibodies on the poly(methyl methacrylate) content of influenza vaccines. Key: ●, polymerization in the presence of the virus; and ▲, polymerization in the absence of the virus.*

[2] Oxford sampler.

Hemagglutination Titers—The hemagglutination titers of the following preparations were determined, using the methods of Hirst (5) and Bonin (6): (*a*) initial fluid virus suspension; (*b*) γ-ray-radiated fluid virus suspension; (*c*) fluid virus suspension containing 0.8% monomeric methyl methacrylate (this mixture was stored at 4° without radiation for 3 weeks); (*d*) poly(methyl methacrylate) particles without virus (plastic control); (*e*) virus suspensions containing various amounts of poly(methyl methacrylate) (Tables I and II and Fig. 1), polymerized in the presence of the virions; and (*f*) virus suspensions containing various amounts of poly(methyl methacrylate) (Tables I and II and Fig. 1), polymerized in the absence of the virions.

In addition to the hemagglutination test of the complete suspensions, the determination of the supernatant fluids of these preparations was carried out after centrifugation at 2000 rpm for 10 min.

Antibody Binding Properties—A 1% albumin solution (0.5 ml) in phosphate-buffered saline was added to 2.0 ml of different poly(methyl methacrylate) adjuvant preparations and to 2.0 ml of the initial virus suspension. The mixtures were shaken vigorously and stored overnight at 4°. Then 0.50 ml of the antibody solution was added, and the mixture was vigorously shaken again. After 1 hr, the polymer particles were removed by centrifugation at 2000 rpm for 10 min. The remaining antibodies in the supernatant fluid were determined by the hemagglutination inhibition test as described by Bonin (7). Polymer particle suspensions without virus served as controls.

The amount of antibodies bound by the polymer–virus preparations was calculated using:

$$\log BU = \log PP - \log AP \qquad \text{(Eq. 1)}$$

Table III—Solubility of Monomeric Methyl Methacrylate

Solvent (100 ml)	Maximum Solubility (20°) of Methyl Methacrylate, ml
Double-distilled water	1.55 ± 0.03
Phosphate-buffered saline, pH 7.2	1.50 ± 0.03
Clear influenza virion suspension	2.00 ± 0.03

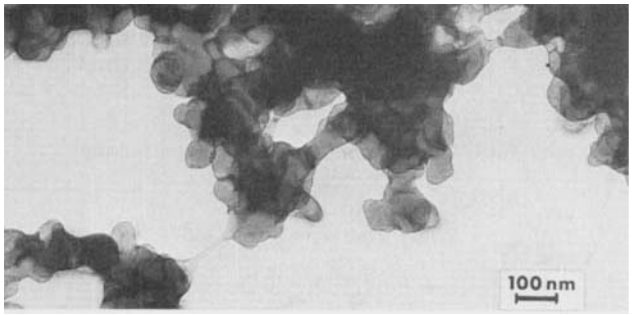

Figure 3—*Influenza vaccine with 0.5% poly(methyl methacrylate) adjuvant polymerized in the presence of the virus. No virions or virion surface structures such as spikes are visible.*

Equation 1 can be transformed into:

$$\log BU = \log \frac{PP}{AP} \qquad \text{(Eq. 2)}$$

where BU = bound antibodies in units, PP = hemagglutination inhibition titer of polymer particles without virus, and AP = hemagglutination inhibition titer of adjuvant preparation.

Transmission Electron Microscopy—The samples were produced by polymerization of 0.5% methyl methacrylate in the presence (Fig. 3) and absence (Fig. 4) of the virions (A2/Aichi, content = 32,000 hemagglutinating units/ml). The virion suspension without adjuvant was examined as a control (Fig. 5). Formvar-coated copper grids were floated on the samples for 3 min. Without removing excess fluid, the grids were brought in contact with 2% phosphotungstic acid for a few seconds. Excess fluid was then removed, and the grids were examined immediately in an electron microscope[3].

RESULTS AND DISCUSSION

Monomer Solubility—Table III shows that about 30% more methyl methacrylate was soluble in the clear virion suspension than in phosphate-buffered saline at pH 7.2. This finding supports the assumption (1) that a certain amount of monomeric methyl methacrylate is adsorbed by the virions.

Hemagglutination Titers—The results of the hemagglutination test are shown in Tables I and II and Fig. 1. Neither addition of the methyl methacrylate monomer and storage for 3 weeks without radiation nor γ-radiation alone decreased the hemagglutination titers of the virus suspension (Table I). These treatments had no changing or damaging effects on the hemagglutinating properties of the virions.

Polymerization in the presence of the virions decreased the hemagglutination titers to a minimum at a poly(methyl methacrylate) concentration of 0.1% and increased the hemagglutination titers at higher poly(methyl methacrylate) contents to the level of the plastic control without virus (Fig. 1 and Table II). This increase above 0.1% poly(methyl methacrylate) was due to prevention of free sedimentation of the erythrocytes by the polymeric particles and thus to simulated hemagglutination.

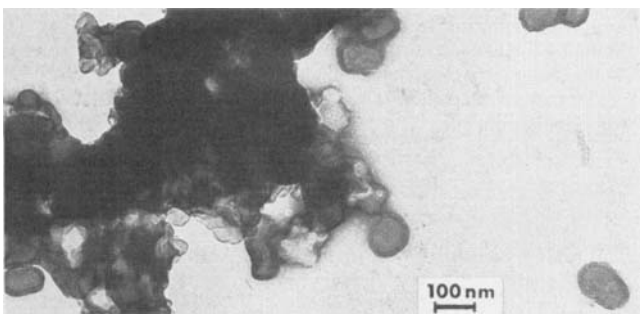

Figure 4—*Influenza vaccine with 0.5% poly(methyl methacrylate) adjuvant polymerized in the absence of the virus. Virions are visible at the surface of the polymer particles as well as independent from the polymer.*

[3] Philips E. M. 301.

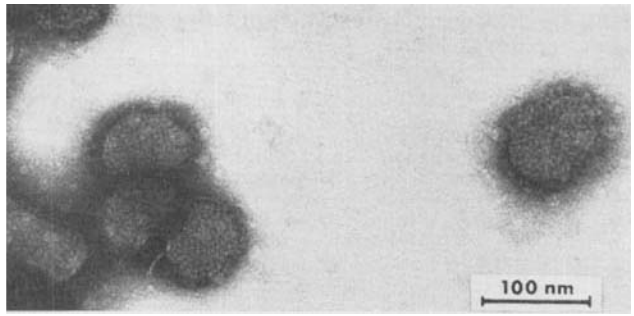

Figure 5—*Initial fluid influenza vaccine without adjuvant (A2/Aichi, content = 32,000 hemagglutinating units/ml).*

If the virions were added to previously polymerized particles, a decrease of the hemagglutination titers was also obtained, although it was much less pronounced (Fig. 1). However, this decrease indicated certain interactions of virions with the plastic particles.

The hemagglutination test of the supernatant fluid after centrifugation of the polymer particles only gave information about the virus that was not associated with the polymer material. A rapid decrease of the hemagglutination activity was obtained with increasing poly(methyl methacrylate) contents (Table II). This decrease again was more pronounced if the polymerization was carried out in the presence of the virions.

Antibody Binding Properties—The antibody binding properties of the poly(methyl methacrylate) adjuvant influenza vaccines gave information about the amount of virus surface not covered by or interacting with polymer. Unbound antibodies could be determined in the supernatant fluid by the hemagglutination inhibition test after removal of the polymer by centrifugation. Therefore, the complication that the polymer particles simulated hemagglutination by preventing the free sedimentation of erythrocytes was avoided. Nonspecific adsorption of antibodies was prevented by addition of 1% albumin.

Figure 2 demonstrates that the amount of antibodies bound by the polymer–virus preparations decreased with an increasing content of poly(methyl methacrylate) in both preparations. However, a much lower amount was bound by preparations polymerized in the presence of the virions than by preparations polymerized in their absence.

Interpretation of Results—These results clearly demonstrate that the products obtained after polymerization in the presence of virus and those obtained after addition of virus to previously polymerized particles were not identical. Moreover, the previous hypothesis (1) that the monomeric methyl methacrylate was adsorbed by the virions and that the virions were coated to a certain extent by the developing polymer during radiation is supported. The electron microscopic picture (Fig. 3) further confirmed this hypothesis: no virions or virion surface structures, such as spikes, can be identified if the polymerization is carried out in the presence of the virions. The extent of coating is dependent on the initial monomer content. Accordingly, increasing contents of the resulting polymer lead to a decrease of virions in the supernatant fluid (Table II) and to a decrease in antibody binding surface (Fig. 2).

As mentioned previously, the virions also interacted with poly(methyl methacrylate) particles prepared by previous polymerization in the absence of virions. These products also decreased the hemagglutination titers and the amount of bound antibodies and yielded a good, but less pronounced, adjuvant effect (1). It can be assumed that adsorption of virions onto the plastic particles took place. Correspondingly, virions were found on the surface of the polymer by electron microscopy (Fig. 4).

The products described here are very similar in size (1) to the "nanoparticles" prepared by Birrenbach and Speiser (8). They produced adjuvants by secondary solubilization of monomeric acrylamide in the presence of tetanus toxoid or γ-globulin in n-hexane and following polymerization. However, the preparation of the products described here is considerably less complicated, thus offering certain advantages over the other procedure.

SUMMARY

1. Monomeric methyl methacrylate was adsorbed by influenza virions in a virus suspension.

2. After polymerization of methyl methacrylate in the presence

of influenza virions, the virions were coated to a certain extent by the polymer.

3. The extent of coating was dependent on the monomer concentration before polymerization.

4. Influenza virions were adsorbed by previously polymerized poly(methyl methacrylate) particles.

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Rapid Spectrophotometric Determination of Salicylamide in Analgesic Tablets

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Abstract □ An independent, simple, and rapid procedure is suggested for the routine analysis of salicylamide in analgesic tablets containing acetaminophen, phenobarbital, caffeine, codeine phosphate, prednisone, ascorbic acid, and chloroquine phosphate. The method does not require the preliminary separation of salicylamide from other constituents by the time-consuming solvent extraction technique or by chromatography prior to determination. The absorbance was linear for investigated concentrations of salicylamide from 0 to 4.0 mg/100 ml of solution at 308 nm.

Keyphrases □ Salicylamide—spectrophotometric analysis in tablets containing other drugs □ Spectrophotometry—analysis, salicylamide in tablets containing other drugs □ Dosage forms—multicomponent analgesic tablets, spectrophotometric analysis of salicylamide □ Analgesics—salicylamide, spectrophotometric analysis in tablets containing other drugs

Salicylamide, an analgesic, antipyretic, and antirheumatic drug, can be determined by visual titration in dimethylformamide against standard sodium methoxide solution with thymol blue indicator (1). Although this method is perfectly suitable for the determination of the drug in pure form, it is totally unsuitable for selective determination in the presence of such acidic substances as acetaminophen, phenobarbital, and codeine phosphate.

Salicylamide can be determined colorimetrically by several methods. The color produced when salicylamide reacts with ferric nitrate in the presence of nitric acid (2) has been the basis for colorimetric determination of the drug after its separation from sodium salicylate and sodium gentisate by extraction with ether. The same reaction was employed for colorimetric determination of salicylamide in serum and urine (3), but after hydrolysis with hydrochloric acid and extraction in ethylene dichloride. Measurements of absorbance were performed at 450 nm. Use of the color resulting from the condensation reaction of salicylamide with *p*-amino-*N*,*N*-dimethylaniline sulfate in the presence of potassium ferricyanide (4) was suggested.

A spectrophotometric procedure was described (5) for the simultaneous determination of five analgesic compounds including salicylamide. Absorbance was measured at three different wavelengths and under three different conditions of acid and base content. Salicylamide also was determined by differential spectrophotometry (6) in the presence of aspirin, acetaminophen, and caffeine. A spectrophotofluorometric method (7) was reported for the simultaneous determination of salicylamide and salicylic acid in blood serum and urine after acid hydrolysis of the salicylamide metabolites.

Salicylamide was determined bromometrically (8) in anhydrous acetic acid by adding aceteous bromine solution and determining unreacted bromine by adding potassium iodide and titrating against thiosulfate.

The objective of this study was to develop a rapid routine analytical method for salicylamide in analgesic tablets containing acetaminophen, phenobarbital, caffeine, codeine phosphate, prednisone, ascorbic acid, and chloroquine phosphate which would be useful for pharmaceutical control purposes.

EXPERIMENTAL

Chemicals—The following were used: salicylamide[1], acetamino-

[1] El-Nasr Pharmaceutical Chemicals Co., A.R.E.