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Research Article

Formulation of Vaccine Adjuvant Muramyldipeptides. 3. Processing Optimization, Characterization, and Bioactivity of an Emulsion Vehicle

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An efficacious vaccine adjuvant which elicits both cell-mediated immunity (CMI) and humoral immune response was developed using [thr[1]]-Muramyldipeptide (MDP) in an oil-in-water emulsion vehicle containing poloxamer 401, polysorbate 80, and squalane. Processing optimization was performed to increase the physical stability of this adjuvant emulsion which, when prepared by conventional mixing methods, demonstrated good bioactivity but poor physical stability. Various manufacturing methods were compared with a microfluidization process, which produced the most stable and elegant emulsion vehicle. The microfluidized emulsion also elicited equivalent biological response in the animal model tested.

KEY WORDS: emulsion processing; emulsion stability; Microfluidizer; muramyldipeptide; adjuvant activity; particle size analysis.

INTRODUCTION

For many years, the only adjuvants approved in the United States for human vaccine use have been aluminum hydroxide and aluminum phosphate. While alum is an adequate adjuvant for use when humoral responses are sufficient to provide protection (e.g., with tetanus toxoid), there is little or no augmentation of cell mediated immunity (CMI) by alum-adjuvanted vaccines (1). In cases where CMI is critical to protection, new adjuvants are required, as well as for those vaccines where the antigen is a relatively poor immunogen (e.g., in some of the subunit vaccines produced by recombinant DNA technology). Freund's complete adjuvant is very effective in induction of both humoral and CMI in experimental animals but is not approved for use in vaccines intended for humans or food animals (2).

A very effective vaccine adjuvant has been developed (3) which contains a muramyl dipeptide analog ([thr[1]]-MDP) (4–6) in an oil-in-water emulsion vehicle. Patents for this formulation, and vaccines prepared with the adjuvant, have been granted or are pending. This vehicle is safe and efficacious, having been used successfully with a variety of antigens in several animal species. The oil-in-water (O/W) emulsion system contains squalane as the oil internal phase and poloxamer 401 and polysorbate 80 as cosurfactants. Poloxamer 401 is a pluronic polyol (L121) exhibiting adjuvant activity (7,8). It functions as a spreading agent (over hydrophobic surfaces, such as squalane) and promotes retention of macromolecules at the oil–water interface (3,9,10). The unique quality of each excipient necessitates their presence in the formulation. The surfactant blend of poloxamer 401 and polysorbate 80 in this formulation has a HLB (hydrophile–lipophile balance) of 1.6. The low HLB implies greater solubility and/or dispersibility in an oil phase and suggests a suboptimal formula for a stable oil-in-water emulsion.

Various processing techniques were evaluated in order to improve the physical stability of this emulsion system. Conventional mixing methods produced unstable emulsions which showed phase separation. However, these preparations exhibited excellent adjuvant activity, as determined by bioassay. One concern throughout the processing studies was that physical changes (such as droplet size or surfactant orientation with regard to the oil phase) of the emulsion might impact on the formulation bioactivity (9). The goal of our processing evaluation was to produce a physically stable emulsion while maintaining the unique balance of the adjuvant and not compromise its bioactivity.

MATERIALS AND METHODS

Reagents. All emulsion excipients were compendial grade and were used without further processing or purification. Each excipient was received as follows: *N*-acetylmuramyl-L-threonyl-D-isoglutamine, Institute of Organic Chemistry, Syntex Research; squalane, NF, Robeco Chemicals; poloxamer 401, NF, BASF Wyandotte Corporation; polysorbate 80, USP, Mazer Chemicals; and ovalbumin and creatine phosphokinase kit 520, Sigma Chemicals.

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Emulsion Preparation. Each adjuvant vehicle was prepared as a 2× concentrate with 10.0% squalane, 5.0% poloxamer 401, and 0.4% polysorbate 80 in 1× phosphate-buffered saline (PBS). Prior to biological testing, the adjuvant vehicle was diluted with an equal volume of a solution containing 0.05% [thr^1]-MDP and 0.2% ovalbumin in 1× PBS. The vaccine was shaken gently to ensure complete mixing. Neither the [thr^1]-MDP nor the ovalbumin was subjected to shearing or potential heating by the various manufacturing methods. Four manufacturing methods were used to optimize physically the adjuvant vehicle; each vehicle was prepared at ambient temperature and the order of excipient addition did not impact on the physical characteristics or the biological activity of the final emulsion. (i) For the standard (vortexing) method, all exipients were combined in a test tube and vortexed vigorously until all visible solid material had dispersed. (ii) For blade mixing, using a modified gate blade and mixer, all emulsion components were mixed at 740 rpm for 40 min. (iii) For homomixing, using a Greerco homogenizer mixer (Model 1L 79, Greerco Corp., Hudson, N.H.), the emulsion components were mixed at approximately 4750 rpm for 30 min. (iv) For microfluidization, the emulsion was cycled four times through a Microfluidizer (Model M110, Microfluidics Corp., Newton, Mass.).

It should be noted that emulsions prepared for biological testing were manufactured under aseptic conditions. Although each emulsion was not evaluated for absence of endotoxin, later studies, evaluating method of manufacture relating to sterility issues, indicated that the manufacturing techniques produced emulsions which passed USP specifications for sterility, LAL, and pyrogenicity.

Particle Size Analysis. The particle sizes of all preparations were characterized by the following techniques. (i) In optical microscopy and visual methods, all of the preparations were photographed using a Leitz Ortholux II POL-BK polarized light microscope (E. Leitz, Inc., Rockleigh, N.J.) at a magnification of 133×. Emulsion particle size was estimated from the photomicrographs using a scale calibrated by a stage micrometer. Visual methods based on Tyndall scattering were useful especially in identifying preparations with submicron particles (these preparations appeared bluish in color).

(ii) In laser photon correlation spectroscopy (PCS), the Nicomp laser particle sizer (Model 200) and a Nicomp computing autocorrelator (Model TC-100, Nicomp Instruments Inc., Goleta, Calif.) were useful for characterizing particle size in the 0.01- to 1-μm range. All the emulsion preparations contained some particles within this size range. With the exception of the microfluidized formulation, sizing was evaluated in the lower portion of the creamed emulsion. Sample dilution was necessary for PCS experiments; control buffer solution (or the emulsion's continuous phase) was used for this purpose. The buffer did not exhibit light scattering, and sample dilution (up to 1:10,000) produced little change in particle size.

(iii) In laser particle size analysis, the emulsion preparations composed of particles greater than 1 μm were evaluated by a Brinkmann particle size analyzer (Model 2010, Brinkmann Instruments Co., Westbury, N.Y.). As above, samples were diluted with the emulsion's continuous phase prior to analysis.

(iv) In freeze-fracture TEM, each emulsion preparations was frozen in liquid nitrogen and freeze-fractured using a Balzars freeze fracture unit (Model 360m, Balzers AG; Furstentun, Liechtenstein) at −115°C, etched for 45 sec at −100°C, and then replica coated. The replicas were recovered easily and cleanly from methanol. Each replica was evaluated on a Zeiss transmission electron microscope (Model EM 109, Carl Zeiss, F.R.G.) operating at 50 kV.

Biological Testing. Immunization and assessment of immune response were performed as described previously (11). The animals used for biological testing were female Hartley strain guinea pigs, weighing 400–450 g, and were obtained from Simonsen Labs (Gilroy, Calif.). Groups of eight animals were injected subcutaneously in the nuchal region with 0.2 ml of the various preparations, so that each guinea pig received 200 μg of ovalbumin and 50 μg of [thr^1]-MDP, in the adjuvant vehicle, for the primary immunization. At 4 weeks, a boost dose containing 50 μg of antigen and no MDP, in 0.2 ml of the adjuvant vehicle, was given. The animals were bled at weeks 4 and 6 by cardiac puncture and also skin tested at week 6 with 10 μg ovalbumin injected intradermally in 0.1 ml saline. To assess delayed hypersensitivity, skin tests were read at both 24 and 48 hr; this was performed by measuring the induration and erythema at the site of injection. Antibody titers were determined by passive hemagglutination (12).

Muscle Irritation Tests. Using groups of five animals, 0.2 ml of each preparation was injected intramuscularly into the thigh muscle of guinea pigs with a 27-gauge needle. After 24 and 48 hr, the injection site was examined for erythema and swelling, and the animals were bled by cardiac puncture. Serum was obtained and stored frozen until creatinine phosphokinase serum assays were performed following the procedure outlined by the phosphokinase kit 520 received from Sigma.

RESULTS

Physical Appearance

The standard (vortexing) method produced an emulsion that appeared physically unstable and displayed rapid (within 1 hr) creaming to form two distinct layers. Due to constraints involving the vortex size and mixing forces, this method was suitable only for test tube size batches of approximately 10 ml. Once the solids (poloxamer 401) had dispersed, additional vortexing did not reduce the internal phase droplet size. The blade mixing method allowed for production of larger batch sizes, but again, the emulsion appeared unstable and creamed within 1 hr. Homomixing produced a relatively more stable emulsion which exhibited some degree of creaming within 24 hr.

The Microfluidizer produced a homogeneous, elegant emulsion displaying little or no creaming. Emulsions are formed in the Microfluidizer by a combination of shear, turbulence, and cavitation forces; two fluidized streams interact at very high velocities within the interaction chamber (13,14). Each cycle of the emulsion through the Microfluidizer decreased the mean droplet size (see Fig. 1). After five passes through the Microfluidizer, the emulsion was com-

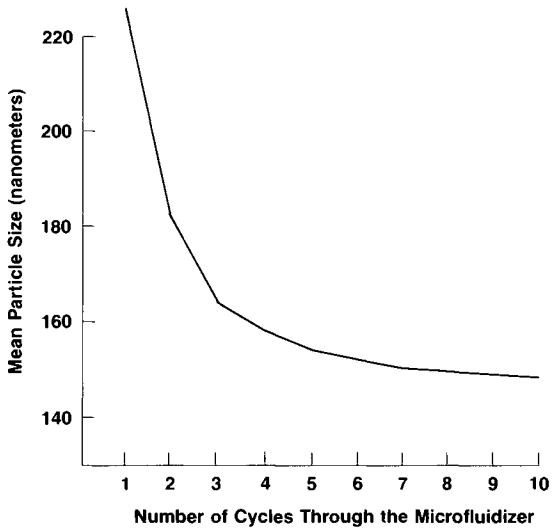

Fig. 1. Size analysis of the submicron particle population as a function of cycles through the microfluidizer.

prised strictly of submicron particles having a mean population size of approximately 150 nm.

Particle Size

Table I summarizes the particle size for each emulsion as determined by the various sizing techniques. Additional sizing data focuses on the standard vortexed and microfluidized preparations because these emulsions represent two size extremes of the four manufacturing methods evaluated. Figure 2 gives laser particle size analysis (Brinkmann) results (probability number and volume density graphs) for the standard vortexed emulsion, and Fig. 3 shows laser photon correlation spectroscopy (Nicomp) distribution results for a microfluidized emulsion sample. Figure 4 presents typical freeze-fracture TEM photomicrographs for the standard vortexed and the microfluidized preparations.

All emulsions exhibited some degree of heterogeneity. The standard vortexed formulation displayed the broadest diversity, whereas the microfluidized emulsion had the narrowest particle size range. The PCS (Nicomp) results show a bimodal size distribution for the microfluidized emulsion: 270 and 90 nm. These size populations were confirmed by the TEM photomicrograph. In order to determine whether one of these populations represented undispersed poloxamer 401, a microfluidized mixture containing 5.0% poloxamer 401 and 0.4% polysorbate 80 in PBS was evaluated by PCS. The results showed that the suspended poloxamer 401 particles were much smaller than either of the two populations

Table I. Emulsion Particle Size Range for Various Manufacturing Methods

Method of Manufacture	Particle Size (μm)
Standard vortexing	0.03–24
Blade mixing	0.01–8
Homomixing	0.02–2
Microfluidizing	0.07–0.2

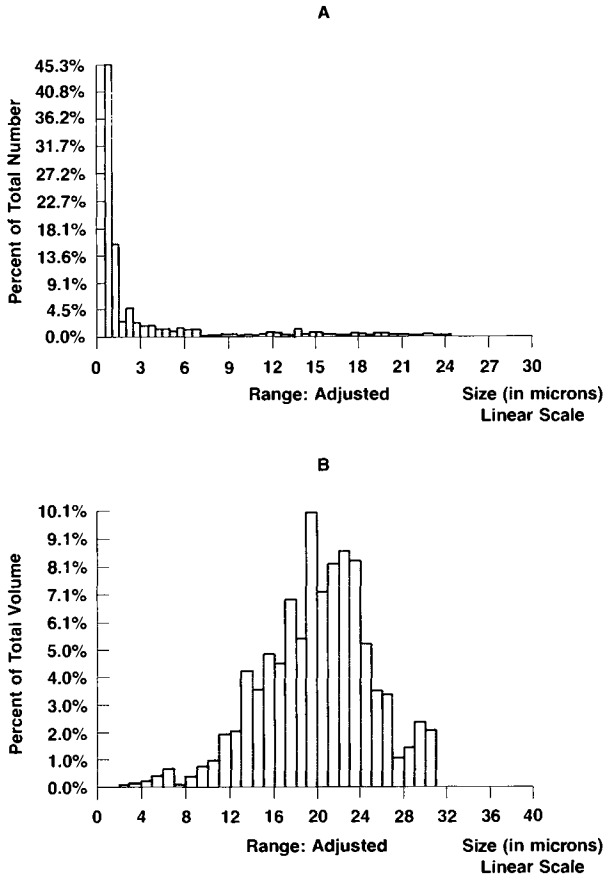

Fig. 2. Brinkmann particle size analysis of the standard vortexed emulsion presented as (A) probability number density graph and (B) probability volume density graph.

observed in the full emulsion. Poloxamer 401 particles were approximately 3 nm.

Laser particle size analysis (Brinkmann) results for the vortexed emulsion are presented in two graphs: probability number density and probability volume density. As a percentage of the total number of particles present in the emulsion, the greatest number of particles are below 2.0 μm in diameter (Fig. 2A). Evaluating the data as a percentage of the total volume, Fig. 2B shows that the greatest distribution is between 12 and 28 μm, with a peak particle diameter at 19.5 μm. The mean particle diameter is 10.1 μm (skewed downward due to the large number of small particles). It should be noted that the microfluidized emulsion cannot be measured by Brinkmann instrumentation because all particles are below 0.5 μm, and the optics of the system will not detect particles below this size.

Biological Testing

Antibody titers and delayed hypersensitivity data are shown in Tables II and III. For all biological testing, vaccines prepared with the standard vortexed emulsion served as controls. All data were compared to the standard within each testing group. Two groups comparing blade mixing and homomixing to the standard method are presented in each table (antibody titer and delayed hypersensitivity). The additional data set is presented because of the biological vari-

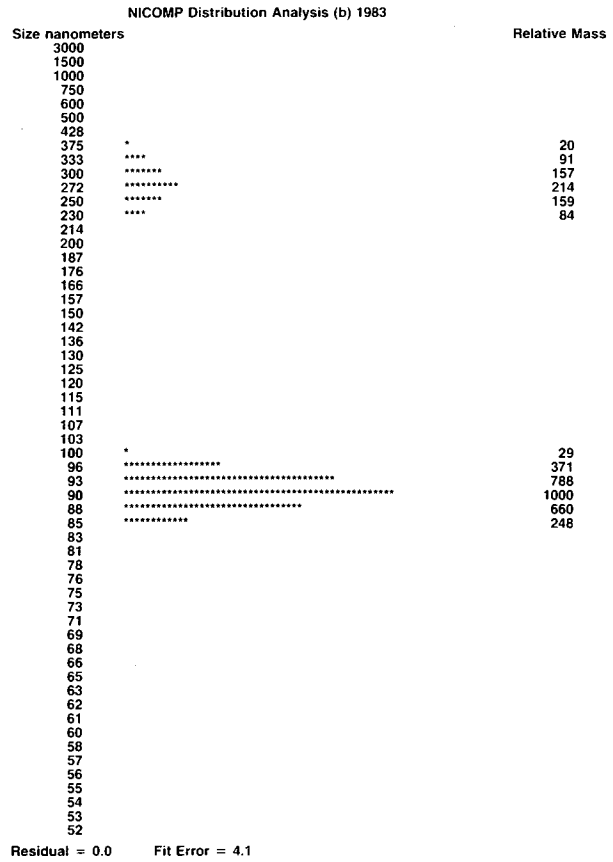

Fig. 3. Particle size analysis of a microfluidized emulsion by photon correlation spectroscopy (Nicomp PCS). Two histograms show the size distribution of the two particle sizes present (272 and 90 nm).

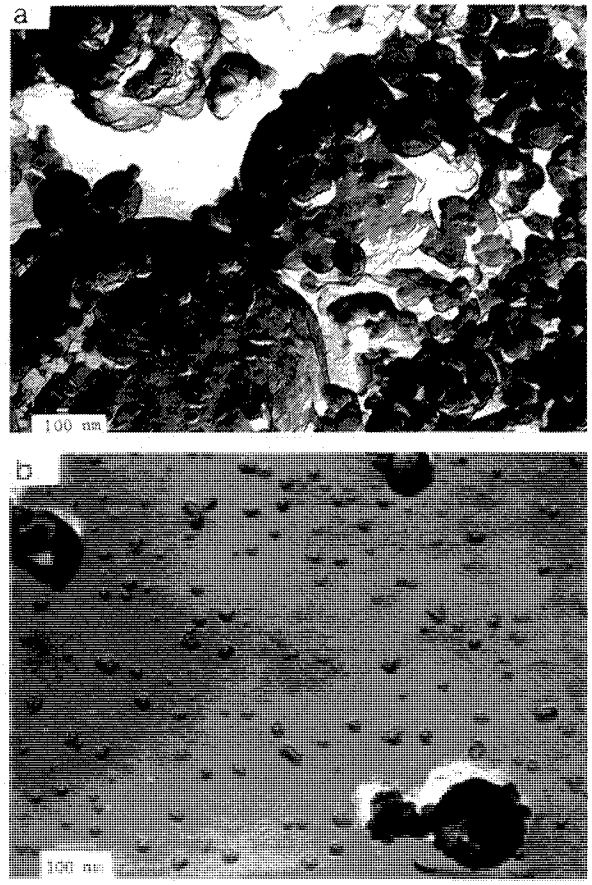

Fig. 4. Transmission electron micrographs of (a) the standard vortexed emulsion (24,320×) and (b) the microfluidized emulsion (84,930×).

ability seen in the data. Overall, the different methods of emulsion preparation generally demonstrated no significant effects on either antibody titer or delayed hypersensitivity skin reactions.

The antibody response to either influenza virus hemagglutinins or hepatitis B virus surface antigen was also evaluated in vaccines containing emulsion which had been either vortexed or microfluidized. The antibody responses to vaccines made with either of the emulsions were similar for both these antigens. As seen with the ovalbumin model, the method of emulsion preparation did not affect the efficacy of these vaccines.

Serum CPK levels are shown in Table IV. The data indicate that the microfluidized formulation was very similar to the standard vortexed formulation in releasing low levels of CPK into the serum following intramuscular injection. Previous experiments have shown that injection of phosphate-buffered saline alone can induce similar serum CPK levels (11). These levels are well below the levels likely to be too irritating for human use (over 3000 IU/liter) (15). Little erythema or swelling at the injection site was observed at 24 or 48 hr, and there were no obvious differences between the injection site reactions of the animals given either formulation.

DISCUSSION

The studies described above have outlined the relationship among processing, physical stability, particle size, and bioactivity of an adjuvant emulsion. These factors are of concern in product manufacture for the purpose of scaleup and determination of useful product life. Understanding product constraints enable well-defined manufacturing considerations.

Table II. Immune Responses to Ovalbumin Comparing Method of Vehicle Manufacture: Antibody Titers[a]

Expt. No.	Group	Titer ± SE	
		28 Days	42 Days
1	Standard	2.25 ± 0.37	6.13 ± 0.30
	Blade mix	1.63 ± 0.18	6.00 ± 0.38
	Homomixed	2.88 ± 0.23	7.00 ± 0.27*
2	Standard	2.63 ± 0.38	7.13 ± 0.23
	Blade mix	2.75 ± 0.37	7.63 ± 0.46
	Homomixed	1.50 ± 0.28*	6.75 ± 0.16
3	Standard	2.13 ± 0.23	7.00 ± 0.27
	Homomixed	2.13 ± 0.35	6.62 ± 0.26
	Microfluidized	1.50 ± 0.19	6.88 ± 0.30

[a] Titer is expressed as $\log_2$ ± standard error of the inverse of the maximal dilution of serum agglutinating egg albumin-sensitized erythrocytes. First dilution of serum was 1/20.

* $P < 0.05$ compared to the standard group.

Table III. Immune Response to Ovalbumin Comparing Methods of Vehicle Manufacture: Delayed Hypersensitivity Evaluated 6 Weeks After Initial Injection

Group	Mean Diameter ± SE (mm) 24 Hr	48 Hr
Standard	16.06 ± 0.62	14.50 ± 0.82
Blade mix	15.00 ± 0.69	10.19 ± 1.74*
Homomixed	17.06 ± 0.47	15.00 ± 0.78
Standard	16.63 ± 1.19	12.94 ± 2.05
Blade mix	17.38 ± 0.77	14.63 ± 0.97
Homomixed	15.43 ± 0.68	12.25 ± 0.42
Standard	18.14 ± 0.34	14.64 ± 0.68
Homomixed	17.31 ± 0.90	11.00 ± 1.84
Microfluidized	19.44 ± 1.06	15.69 ± 0.87

* $P < 0.05$ compared to the Standard Group.

These studies demonstrated that emulsion particle size is reduced by increasing the shear forces in the mixing process. Greater emulsion stability, as evidenced by a decreased tendency for creaming, was a benefit from the decreased particle size distribution (16). Increased stability of the microfluidized emulsion may be attributed to greater mixing efficiency between poloxamer 401 and squalane. The emulsion produced by this manufacturing method was by far the most robust; extended centrifugation of this sample failed to break the emulsion. As shown by the various sizing techniques, the microfluidized emulsion is homogeneously submicron and has a population size distribution which is at least 5- to 10-fold smaller than the other emulsions tested. The microfluidized emulsion, with its small particle size, appeared to be the most physically elegant emulsion, demonstrating ease of manufacture with a high degree of reproducibility. These attributes lend themselves to commercial viability for this parenteral emulsion.

The vortexing method of emulsion preparation, which is suitable only for making small experimental batches, was used in the initial development of the adjuvant formulation (11). Immune responses of animals immunized with vaccines manufactured by methods having potential for large-scale production were compared to responses of animals given vaccines made with the vortexed emulsion. An important issue was whether use of emulsions which displayed different particle sizes would result in vaccines with different bioactivities, since emulsion particle size could affect binding of antigen to emulsion particles.

Table IV. Serum Creatinine Phosphokinase Levels Following Intramuscular Injection of Emulsion Vehicle[a]

Emulsion	Mean IU/liter CPK ± SE 24 Hr	48 Hr
Standard vortexed	321 ± 28	220 ± 59
Microfluidized	372 ± 52	159 ± 23

[a] CPK levels were measured in guinea pig sera following intramuscular injection of emulsion excluding [thr^1]-MDP.

All emulsions were physically distinct with regard to particle size distribution. Yet no significant differences were observed for the emulsions when comparing antibody titer and delayed hypersensitivity. In the model system used for these studies, oil phase globule size appears not to affect bioactivity of this adjuvant emulsion. Antibody response may not be contingent upon emulsion stability or globule size; biological response may occur as long as an emulsion is formed (17).

New vaccine adjuvants must be safe, efficacious, and easy to use. Simple, efficient, and reproducible large-scale manufacturing methods are also desirable. By producing an elegant and stable emulsion in a rapid and reproducible fashion, the Microfluidizer meets all these requirements.

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REFERENCES

1. R. Bomford. *Clin. Exp. Immunol.* 39:435 (1980).
2. A. C. Allison, N. E. Byars, and R. V. Waters. In R. M. Nervig, P. M. Gough, M. L. Kaeberle, and C. A. Whetstone (eds.), *Advances in Carriers and Adjuvants for Veterinary Biologics,* Iowa State University Press, 1986, pp. 91–103.
3. A. C. Allison and N. E. Byars. *J. Immunol. Meth.* 95:157–168 (1986).
4. M. F. Powell, L. C. Foster, A. R. Becker, and W. A. Lee. *Pharm. Res.* 5:528–532 (1988).
5. G. Jones, J. Moffat, and J. Nestor. U.S. Patent 4,082,735 (1978).
6. G. Jones, J. Moffat, and J. Nestor. U.S. Patent 4,082,736 (1978).
7. R. L. Hunter. In B. H. Tom and H. Six (eds.), *Liposomes in Immunobiology,* Elsevier North-Holland, 1980, pp. 25–38.
8. H. Snippe, M. J. Reuver, F. Strickland, J. M. N. Willers, and R. L. Hunter. *Int. Arch. Allergy Appl. Immunol.* 65:390–398 (1981).
9. R. Hunter, F. Strickland, F. Kezdy. *J. Immunol.* 127:1244–1250 (1981).
10. R. L. Hunter and B. Bennett. *J. Immunol.* 133:3167–3175 (1984).
11. N. E. Byars and A. C. Allison. *Vaccine* 5:223–228 (1987).
12. A. B. Stavitsky. *J. Immunol.* 72:360 (1954).
13. H. Korstvedt, G. Nikolopoulos, S. Chandonnet, and A. Siciliano. *Am. Paint Coat. J.* Jan. 28:38–40 (1985).
14. E. Mayhew, R. Lazo, W. J. Vail, J. King, and A. M. Green. *Biochim. Biophys. Acta* 775:169–174 (1984).
15. J. E. Gray, R. N. Weaver, J. Moran, and E. S. Feenstra. *Toxicol. Appl. Pharmacol.* 27:308–321 (1974).
16. M. Singh and L. J. Ravin. *J. Parent. Sci. Tech.* 40:34–41 (1986).
17. L. F. Woodard and R. L. Jasman. *Vaccine* 3:137–144 (1985).