Improved Technique for the Preparation of Water-in-Oil Emulsions Containing Protein Antigens

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Water-in-oil emulsions are commonly used to provide persistent release of antigens necessary for sustained stimulation of the immune system. Optimal response of an animal’s immune system is dependent upon repeated, maintained exposure to the antigen. The sustained release of antigen, which delays catabolism, is thought to be one of the major methods by which adjuvants enhance the immune response. Water-in-oil emulsions are also used to provide sustained release of pharmaceutical agents and hormones.

The most frequently used method for preparing a water-in-oil emulsion employs the “two-syringe” technique (1,3). The two syringes are connected with a double-ended Luer-lok® (Becton Dickinson, Lincoln, NJ, USA) connector (or three-way valve). The emulsion is generated by forcing a mixture of oil and water back and forth until the material becomes opaque white, homogeneous and viscous. The mixing is continued until emulsion thickens and it becomes difficult to extrude the material through the connector. Although the two-syringe method is conceptually very simple and requires only standard laboratory equipment, we have experienced difficulty producing durable water-in-oil emulsions using this technique. The emulsion produced by this technique is very sensitive to temperature of the antigen and adjuvant solutions, presence of detergent and many other factors including physical properties of the antigen. Many times it does not result in a stable water-in-oil emulsion, and it may produce an oil-in-water emulsion, which will disperse when tested.

Our laboratory uses a different technique to produce a water-in-oil emulsion that is a modification of the method suggested by Crowle (2). This technique yields a durable emulsion with excellent stability when tested in water or physiological saline. The emulsion can be produced in samples that contain small amounts of nonionic detergents. In addition, most of the sample is recovered during preparation, an advantage when dealing with small amounts of valuable protein antigen. The only equipment required, in addition to standard laboratory equipment, is that of a variable-speed Dremel Moto tool (or equivalent), which is available at most hardware stores for about $50.

Figure 1. Addition of the antigen solution to the adjuvant oil. The nail mounted in a variable-speed Dremel Moto tool is immersed in the adjuvant solution while spinning at 75% of maximal velocity. The antigen solution is added dropwise to form an emulsion. To ensure complete suspension after the antigen is added, the Moto tool is operated at full speed for 15–20 s.
Benchmarks

to $70, and a box nail. This technique has been used to produce monoclonal antibodies directed against tyrosine hydroxylase (4) and tau protein. Some of these monoclonal antibodies directed against tau protein have been demonstrated to be dependent upon the presence of a phosphate group in the primary epitope. In addition, this technique has been used to produce highly specific polyclonal antibodies in rabbits directed against tyrosine hydroxylase (5).

The equipment needed for this technique is as follows: a variable-speed Dremel Moto tool or equivalent; two-and-a-half-inch 8-penny box nail; sterile container (24-well tissue culture plate); sterile 19- to 21-gauge needles; Complete or Incomplete Freund’s adjuvant (Sigma Chemical, St. Louis, MO, USA); and antigen in phosphate-buffered saline (or most other aqueous solutions).

The procedure is as follows: assemble the Moto tool on a stand with a clamp. Put the nail in the chuck and tighten as per manufacturer’s directions. THE USE OF SHATTERPROOF EYE PROTECTION IS STRONGLY RECOMMENDED. Place 1 mL of adjuvant solution or mineral oil into selected wells of the sterile tissue-culture plate. Hold the tube with the nail immersed in the adjuvant as shown in the diagram and turn the Moto tool to approximately 75% of maximal velocity. Add approximately 1 mL of the antigen solution to the well dropwise over a period of approximately 30 s using a Pasteur pipet. The mixture should turn cloudy and start emulsifying as the antigen is added. After all of the antigen is added, move the nail around in the mixture to ensure complete mixing. Finally, turn the Moto tool to full speed for an additional 15 to 30 s to ensure complete mixing. Turn off the Moto tool and examine the emulsion. It should be very viscous and leave an indentation where the nail had been.

The emulsion can be transferred to a 1-mL syringe by slowly aspirating the viscous mixture through the hub. Any air that is aspirated into the syringe is removed by gently tapping the inverted syringe body with a hard instrument (a pair of scissors) since the emulsion is quite viscous and will slowly flow down to the plunger. The plunger is slowly advanced while tapping until no air remains in the syringe.

Since the emulsion is being prepared to inject into animals, the durability of the emulsion should be tested under physiological conditions to ensure integrity of the water-in-oil emulsion. A small amount of the emulsion is placed on the surface of a beaker of physiological salt solution (either phosphate or Tris-buffered saline) at room temperature or 37°C. However, we routinely use distilled water at room temperature. In either case, the emulsion should remain intact and not disperse for a long period of time. In our hands, emulsions prepared by this method using a variety of antigen solutions have remained intact for up to a week at room temperature.

The chemistry of forming emulsions is complex and many factors can affect
the quality of the emulsions. Therefore, when using a valuable antigen, we first use a test buffer mix containing all of the components except the antigen. If a satisfactory emulsion is produced, then we repeat it with the antigen present. We have found that this technique will work in the presence of small amounts of nonionic detergent (Nonidet® P-40 and Triton® X-100) and various buffers including Tris and phosphate, and it will work with completely homogenized sodium dodecyl sulfate (SDS) gel slices. Again, when using a new system of antigens and buffers, test it first. We have also found that varying the ratio of adjuvant to antigen solution between 1:1 and 1:2 will result in the production of a satisfactory emulsion.

Since most antigen is prepared for injection into multiple animals, the amount of antigen injected into each animal can be easily determined by simply weighing the syringe. The syringe is weighed after loading and testing of the emulsion. It is weighed again following the injection of each animal, thereby more accurately determining the amount of antigen injected into each animal, and this allows an even division of the antigen between the number of animals to be injected.

This procedure should work with most mineral oils that are usually used as adjuvants. However, the author has only used mineral oils that are commercially available as adjuvants.

REFERENCES


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