Importance of Phosphate in Freund Adjuvant[1] (36445)

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Freund adjuvant is an emulsion of water-in-oil (1) which has become an essential and widely used reagent in biological experimentation because of its unequaled capacity to stimulate immunologic responses. But because its mode of action is poorly understood, its use still is largely empirical (2). Its physicochemical characteristics, and the biologic properties of the mycobacteria often added to it, account for some of its effects, such as piecemeal prolonged release of antigen from the injection depot (3, 4), distribution of antigen body-wide by the lymphatic system (5), attraction of antibody-making cells to the depot (6), and enhanced local enzymatic digestion of antigen (7). The roles of oil, surfactant, and mycobacteria in the adjuvant have been frequently investigated and have been partially elucidated. But the effects of changing the composition of its aqueous phase have not, because there has been no reason hitherto to suspect that these would be important.

Some investigators (8, 9) have been unable to induce delayed-type hypersensitivity in mice to purified acidic (anionic) proteins injected in incomplete Freund adjuvant (IFA; *i.e.*, water-in-oil emulsion lacking mycobacteria), whereas we have succeeded in doing so routinely (10–12). Recently, we have discovered that this discrepancy may be due to their having used saline alone for making IFA, whereas we have been using a phosphate-buffered salt solution. The results supporting this conclusion are reported here.

Materials and Methods. CAF_1 female mice (Jackson Memorial Laboratory, Bar Harbor, ME) 6 to 10 weeks old were used, usually in groups of 10; they were allowed unlimited quantities of water and commercial mouse food. Four-times crystallized ovalbumin (OVA; Nutritional Biochemicals Corp., Cleveland, OH) and crystallized human serum albumin (HSA; Pentex, Kankakee, IL) were used as antigens. IFA was prepared with *n*-hexadecane (Practical) and glycerol monooleate ("Myverol"), both obtained from Distillation Products Industries, Rochester, NY. The IFA was composed of *n*-hexadecane:glycerol monooleate:water = 4:1:10 (10, 11). Sodium phosphate-buffered saline, physiologic saline, and saline buffered with Tris or with sodium barbital were mixed according to specifications in standard formula tables (13) using reagent-grade chemicals and freshly distilled water. Pyrogen-free injectable saline was purchased from Travenol Laboratories, Inc. (Morton Grove, IL). Pyrogen added to IFA for one of the experiments was *Salmonella typhimurium* lipopolysaccharide B (Difco Laboratories, Detroit, MI).

Our experimental model has been described in detail before (10, 11). Briefly, mice were sensitized by injecting them subcutaneously on days 0 and 7 with 0.1 ml volumes of IFA containing 250 or 10 μg of antigen. They were skin-tested at 3, 5, and 7 weeks with 20 μg quantities of the same kind of antigen used for sensitization dissolved in 0.02 vol of physiologic phosphate buffer. Arthus (3-hr) and delayed hypersensitivity (24-hr) reactions to these skin tests were measured with calipers in diameter and thickness (10). Since no equivocal readings were obtained (e.g., the lowest positive reading was 7 mm, and highest negative was less than 2 mm in diameter), results are recorded here simply as percentage positive for a given reading and group of mice. To further

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TABLE I. Contrast Between Saline and Physiologic Sodium Phosphate Buffer in Constituting a Sensitizing IFA with HSA.

Immunization	5-Week reactions[a]	
	Arthus	DTH
1. 10 μg HSA, phosphate	100	90
2. 10 μg HSA, saline	90	10
3. 250 μg HSA, phosphate	89	89
4. 250 μg HSA, saline	80	0

[a] Percentage positive.

simplify the data, only results from the 5-week tests, when delayed hypersensitivity peaks (10), are presented. Results from the 3- and 7-weeks tests agreed with and supported those of 5 weeks.

Results. Our first experiment (Table I) illustrates the inefficiency of saline as contrasted with physiologic sodium phosphate buffer to constitute a delayed hypersensitivity-inducing IFA in mice with either of two doses of HSA. Arthus hypersensitivity was induced well by both types of aqueous phase.

This inefficiency of the saline was not associated with a lack of pyrogen (14). Saline to which pyrogen was added for each immunizing injection to contain 10 μg was no better than pyrogen-free saline for sensitizing to either OVA or HSA (Table II).

A quantity dependency for phosphate ions is suggested by results from Table III comparing pyrogen-free saline, freshly made saline, phosphate buffer (pH 7.4) at ionic strength 0.05, and a 1:1 mixture of this buffer and saline. This effect might be due as much to pH regulation as to the presence of phosphate ions. But results from an additional experiment (Table IV) comparing ionic strength 0.15 sodium phosphate buffer at three different pH values, and equal-strength buffers at pH 7.4 prepared with Tris or with sodium barbital instead of sodium phosphate indicate that the presence of phosphate ions is paramount. Nevertheless, the adjuvancy of phosphate ions does seem to be optimized by their use at near-neutral pH.

Discussion. We do not understand how phosphate ions contribute to the adjuvancy of IFA and therefore can only offer a speculative explanation. Basic (cationic) proteins, and other antigens which tend to combine spontaneously with host tissue, readily induce delayed-type hypersensitivity in mice when injected in either distilled water- or saline-constituted IFA (15–17). Cell membranes, especially of macrophages, participate importantly as complexes with antigen in inducing both humoral antibody and delayed hypersensitivity responses (18, 19). Since we find the effect of phosphate ions to be both concentration- and pH-dependent, this effect may result from changing the mode or locale of antigen attachment to (or ingestion by) (20) macrophages from that which occurs with saline, and favors induction of only humoral antibody responses, to another which promotes induction of delayed hypersensitivity in addition to humoral antibody production. Since this effect probably is not due to direct interaction between phosphate ions and antigen (21), most probably it affects either the host cells or antigen interaction with these. A difference in mechanisms for induction of Arthus and of delayed hypersen-

TABLE II. Effect of Pyrogen Added to the Aqueous Phase of IFA on Sensitization.

Immunization	5-Week reactions	
	Arthus	DTH
1. 10 μg OVA in phosphate	100	56
2. 10 μg OVA in saline	50	0
3. 10 μg OVA in saline–pyrogen	100	0
1. 10 μg HSA in phosphate	80	30
2. 10 μg HSA in saline	100	0
3. 10 μg HSA in saline–pyrogen	90	10

TABLE III. Effect of Phosphate Ions in Aqueous Phase of IFA on its Sensitizing Capacity.

Immunization	5-Week reactions	
	Arthus	DTH
1. 250 μg HSA in pyrogen-free saline	90	0
2. 250 μg HSA in phosphate	100	100
3. 250 μg HSA in saline	90	0
4. 250 μg HSA in saline: PO_4^{3-} = 1:1	100	30

TABLE IV. Effect of Changing pH or Buffer Composition on Aqueous Phase of IFA on Sensitization.

Immunization	5-Week reactions	
	Arthus	DTH
1. 250 μg HSA in saline	90	0
2. 250 μg HSA in pH 7.4 phosphate	100	50
3. 250 μg HSA in pH 8.0 phosphate	77	9
4. 250 μg HSA in pH 6.4 phosphate	100	0
5. 250 μg HSA in pH 7.4 Tris	90	0
6. 250 μg HSA in pH 7.4 barbital	10	0

sitivities is implied by the equally good Arthus sensitization effected by all varieties of IFA tested.

Whatever these results mean mechanistically, they help explain the inability of some laboratories to induce delayed hypersensitivity in mice to purified proteins injected in IFA, they indicate a necessity for additional study of the role of the aqueous phase of IFA in inducing hypersensitivity, and they suggest that for at least some forms of experimentally induced delayed hypersensitivity IFA can be used instead of complete Freund adjuvant if it contains an adequate supply of phosphate ions.

Summary. The induction of delayed hypersensitivity in mice to purified acidic (anionic) proteins by injecting these subcutaneously in incomplete Freund adjuvant appears to require the aid of phosphate ions in the aqueous phase of this adjuvant. Arthus hypersensitivity to these antigens is readily induced by such injections whether phosphate ions are present or not. Phosphate may function by affecting host cell–antigen associations in the earliest stages of induction. These experiments suggest that a more detailed investigation of the functions of the aqueous phase of Freund adjuvants is needed, and also that for at least some models of delayed hypersensitivity incomplete Freund adjuvant can be used in place of complete Freund adjuvant if phosphate ions are present.

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