Carbonyl Iron in Humoral Antibody Responses[1] (36271)

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Diverse particulate and colloidal materials including aluminum phosphate, silica, paraffin gels, and india ink have been used as adjuvants in immune systems (1–5). Some of these substances apparently act by retention or adsorption of antigen (4, 5); whereas others act by the induction of reticuloendothelial cell proliferation (6).

Certain iron preparations have also been used successfully, but their mode of action is unclear (7–9). In view of the interest in elucidating the mechanisms of adjuvants and because of the special physical and immunological properties of carbonyl iron (metallic iron of small spherical particles) (9), the effects of this substance on the humoral antibody response to a cellular, a micellar, and a soluble antigen were investigated.

Materials and Methods. Rats were obtained from Microbiological Associates, Inc., Bethesda, MD, Camm Research Institute, Wayne, NJ, or bred in our laboratories, and were maintained on Purina Laboratory Chow and water. Intravenous (iv) injections were made in the dorsal penile vein and bleedings were from the retroorbital venous plexus. Carbonyl iron SF was donated by GAF Corp., Linden, NJ, through the courtesy of T. Tarantino. The other particulate agents included kaolin (Peerless Clay No. I, donated by R. T. Vanderbilt Co., Inc., New York, NY), polystyrene DVB (No. 6B cation exchange and anion exchange resins (spherical particles, 2–5 μ diam, from Particle Information Service, Los Altos, CA).

Antisera. Sheep red blood cells (SRBC) in Alsever's solution were washed 4–5 times with normal saline or 0.15 *M* Tris buffer, pH 7.4. If no lysis occurred in the final wash, the cells were standardized to a 1% suspension in saline according to Campbell *et al.* (10). Lewis female and (L $\times$ BN) Fl hybrid male rats were injected as outlined in Table I. A 10% suspension of carbonyl iron in saline was the adjuvant.

Bovine serum albumin (BSA, Armour Pharmaceutical Co., Chicago, IL) in saline was injected into Wistar rats at 10 mg of BSA each, and into Lewis rats at 0.5 mg each as outlined in Table II. The adjuvants were complete Freund's adjuvant [CFA (9)] and 10% carbonyl iron in saline.

Horse ferritin (Pentex, Inc., Kankakee, IL) was purified and sterilized according to Andres *et al.* (11). The adjuvants included:

1. 5% carbonyl iron in distilled water.
2. 1% polystyrene cation exchange resin in 0.005 *M* phosphate buffer, pH 7.2
3. 1% polystyrene anion exchange resin in distilled water
4. 2% polystyrene anion exchange resin in distilled water.
5. 5% kaolin in phosphate buffer
6. CFA

Ferritin was added to suspensions 1–3 and 5 to give a final concentration of 1.75 mg/ml for ip inoculation, and to suspension 4 at 3.5 mg/ml for iv inoculation. Suspensions 1–4 were incubated at 37° for 30 min; whereas suspension 5 was incubated at 50° for 10 min and then cooled. In addition, 1 vol of ferritin was emulsified in 1 vol of CFA to give a final ferritin concentration of 0.75 mg/0.05 ml of emulsion. Lewis male and female rats were then injected as outlined in Table III.

Titration of antibodies. Antibody titers were the reciprocals of the highest dilutions

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showing visible agglutination after overnight incubation at 4°.

Direct hemagglutination of SRBC was carried out with sera inactivated at 56° for 30 min. Twofold serial dilutions of each serum were made to a final volume of 0.5 ml with 0.1% BSA in Tris buffer (0.15 *M*, pH 7.4). Then 0.05 ml of 2% SRBC in Tris buffer was added to each tube. The tubes were shaken intermittently at room temperature for 1 hr before overnight incubation.

SRBC hemolysins were titrated according to Campbell *et al.* (10), using the equation of Von Krogh (12) to determine the 50% end point.

BSA. Passive hemagglutination with the Microtiter equipment from Cooke Engineering Co., Alexandria, VA, was carried out with BSA-coated rabbit erythrocytes, stabilized by formaldehyde and pyruvic aldehyde (FPRE), according to the method of Hirata and Brandriss (13). Plastic plates with multiple "V" bottom wells were used for sharper end points. Each antiserum was absorbed for 1 hr at 37° with 0.5 vol of uncoated FPRE before twofold serial dilutions were made to a final volume of 0.025 ml with 1% normal Lewis rat serum in phosphate buffer (0.1 *M*, pH 7.2) as the diluent. Equal volumes of 0.25% BSA–FPRE in phosphate buffer were added to the serially diluted samples. The plates were shaken at 10 min intervals for 30 min at room temperature and then allowed to stand at 4° overnight.

Ferritin. Passive hemagglutination with the Microtiter system was carried out with ferritin-coupled formalinized SRBC. The technique of Butler (14) utilizing bis-diazotized benzidine as coupling reagent was used. Tris buffer containing 10% normal Lewis rat serum was the diluent; and 0.25% formalinized ferritin–SRBC was the indicator.

Results. Antibodies to SRBC. Lewis and LxBN rats produced low titers of antibodies after ip or iv injection of SRBC without adjuvants. When carbonyl iron was added to SRBC, the red cells became clumped to the iron particles. The ip inoculation of this suspension resulted in an enhanced antibody response (Table I). However, a similar enhancement was found when the iron was in-

TABLE I. The Effects of Carbonyl Iron on the Antibody Response to SRBC in Rats.

Strain of rat	Antigen, adjuvant, and route of injection: ip	iv	Log₂ reciprocal of mean titer — Agglutinins: 6[c]	10	14	22	29	Hemolysins: 6	10	14	22	29
Lewis[a]	1 ml SRBC–1 ml iron	—	—	12.0	11.3	12.4	11.6	—	8.0	7.9	7.5	6.9
	1 ml SRBC–1 ml saline	—	—	6.3	7.2	6.3	6.3	—	—	3.2	3.8	2.6
	1 ml iron	1 ml SRBC	—	13.1	10.6	11.2	10.8	—	8.1	7.9	6.8	6.7
	1 ml saline	1 ml SRBC	—	9.7	6.7	7.3	6.7	—	4.4	2.8	<1.0	<1.0
	1 ml iron–1 ml saline	—	—	<3.3	<3.3	<3.3	<3.3	—	<1.0	<1.0	<1.0	<1.0
	2 ml saline	—	—	<3.3	<3.3	<3.3	<3.3	—	<1.0	<1.0	<1.0	<1.0
L × BN[b]	1 ml SRBC–1 ml iron	—	11.9	13.0	12.7	—	11.6	9.6	8.9	8.6	—	8.4
	1 ml SRBC–1 ml saline	—	7.6	9.1	8.8	—	7.9	7.9	6.1	5.0	—	3.6

[a] Three rats per group.
[b] Four rats per group.
[c] Days after injection.

TABLE II. The Effects of Carbonyl Iron on the Antibody Response to BSA in Rats.

Strain of rat	Antigen, adjuvant, and route of injection: ip	iv	Footpad	Log₂ reciprocal of mean agglutinin titer: 6[c]	10	13/14	20/22
Lewis[a]	1 ml BSA–1 ml iron	—	—	4.8	5.6	6.2	5.4
	1 ml BSA–1 ml saline	—	—	<1.0	<1.0	<1.0	<1.0
	1 ml iron	1 ml BSA	—	<1.0	<1.0	<1.0	<1.0
	1 ml saline	1 ml BSA	—	<1.0	<1.0	<1.0	<1.0
	1 ml iron–1 ml saline	—	—	<1.0	<1.0	<1.0	<1.0
Wistar[b]	1 ml BSA–1 ml iron	—	—	3.6	—	4.6	4.0
	1 ml BSA–1 ml saline	—	—	3.0	—	1.0	3.2
	1 ml iron	—	0.05 ml BSA	3.6	—	3.0	3.6
	—	—	0.05 ml BSA	2.0	—	2.6	2.6
	—	—	0.05 ml BSA–CFA	8.6	—	10.6	11.6

[a] Five rats per group.
[b] Two rats per group.
[c] Days after injection.

jected ip and the SRBC were injected separately., iv. Both hemolysins and agglutinins persisted when iron was used; whereas in its absence, the hemolysin production from iv injection of SRBC fell quite rapidly.

Antibodies to BSA. The adjuvant activity of iron for BSA was very small compared to the effect of CFA (Table II). The adjuvant activity in Lewis rats could be observed only when iron was injected by the same route as BSA, but larger doses of BSA injected into Wistar rats resulted in a slight increase of antibody whether antigen and adjuvant were given by the same or different routes.

Antibodies to ferritin. Optical density measurements of supernatants at 280 mμ showed that ferritin was adsorbed completely on anion exchange resin, about 70% on kaolin, 27% on cation exchange resin, and only about 4% on the carbonyl iron.

All these agents had adjuvant activity for ferritin in Lewis rats (Table III). The order of decreasing adjuvant activity was: CFA $>>$ anion exchange resin $\geq$ kaolin $>$ cation exchange resin $>$ carbonyl iron. CFA was over 200 times as effective as kaolin or the anion exchange resin; whereas the latter two agents were over 30 times as effective as carbonyl iron.

The adjuvant effect of CFA differed signifi-

TABLE III. The Effect of Different Adjuvants on the Antibody Response to Ferritin by Lewis Rats.[a]

Antigen, adjuvant, and route of injection: ip	iv	Footpad	Log₂ reciprocal of mean agglutinin titer: 6[b]	14	21	29
2 ml ferritin–iron	—	—	8.3	8.3	7.3	7.4
2 ml ferritin–cation exch. resin	—	—	7.6	8.6	9.1	11.1
2 ml ferritin–kaolin	—	—	9.8	13.1	13.3	12.7
2 ml ferritin–anion exch. resin	—	—	9.8	13.6	13.6	13.6
—	1 ml ferritin–anion exch. resin	—	10.8	10.0	9.3	—
—	1 ml ferritin	—	6.3	4.0	4.0	—
2 ml ferritin	—	—	4.5	4.7	5.1	5.1
—	—	0.05 ml ferritin–CFA	14.7	17.7	19.7	22.0

[a] Five rats per group.
[b] Days after injection.

cantly from that of kaolin and the anion exchange resin not only in the amount of antibody elicited but also in the kinetics of antibody production. When CFA was used, antibody production increased from 6 days to at least 29 days after injection. On the other hand, antibodies after ip injection of antigen with kaolin or ion exchange resins leveled off at 14 days and remained relatively constant to 29 days.

Discussion. Carbonyl iron had a strong adjuvant effect for SRBC. It had less effect with BSA and ferritin, and was distinctly inferior to CFA for these antigens.

In 2 of 3 experiments, the iron was effective when given by a route different from that used for the antigen (Tables I and II). This suggests a mechanism of systemic stimulation of the reticuloendothelial system.

However, local effects may be involved also, as was found in previous work with whole tissue antigens (9). SRBC were noted to clump around iron particles when mixed together. Similarly, ferritin was found to adsorb to iron and other particulate materials. Most impressive was the observation that the adjuvant potency of various particulates was proportional to the degree of adsorption of the ferritin antigen (Table III). An intimate relation of adjuvant and antigen could enhance antibody production by facilitating absorption, dissemination and processing of antigen, by acting as a depot at site of inoculation and draining lymph node, and by stimulating a favorable cellular proliferation.

Local and systemic effects of iron and other particulate adjuvants are not necessarily completely separable. Both antigen and adjuvant are widely disseminated after injection by separate routes, and they may meet in lymph nodes and spleen to create effects identical to those invoked for combined injections.

Particulate adjuvants may also protect antigens from premature degradation. Inasmuch as adjuvant particles are present in excess, it is possible that they blockade peritoneal macrophages, and thereby postpone digestion of antigen and permit more of it to reach the spleen, as described by Perkins (15).

Summary. Carbonyl iron (metallic iron of small spherical particles) had strong adjuvant effect for antibodies to sheep red blood cells in rats. It had a lesser effect with bovine serum albumin and ferritin, and was distinctly inferior to Freund's adjuvant for those antigens. Both local and systemic factors contributed to the adjuvancy of carbonyl iron.

The adjuvancy of a series of particulate agents toward ferritin was proportional to the degree of adsorption of this antigen on the particles.

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