Antigen Distribution in a Latex Suspension and Its Relationship to Test Sensitivity

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Specific aggregation of latex particles due to antigen-antibody reaction occurred only when antigen was latex bound, as found in a study using *Escherichia coli* 0111:B4 extract. Maximum serological sensitivity was achieved when free antigen was present in a narrow range of ratios with the bound antigen.

Latex agglutination is a complex mechanism that is not yet fully understood. Usually antigen (Ag) exists in the latex-Ag reagent in two physical states: free in solution (FAG) and bound to latex (BAG). Bound Ag particles aggregate with the corresponding antibody both in the presence (9) and in the absence (1) of FAG. When Ag is prevented from adsorbing to the latex, it reacts with antibody in solution, resulting in agglutination of uncoated particles (2).

In a previous report (5) we have shown that when an ethylenediaminetetraacetate extract of *Escherichia coli* Ag is adsorbed on latex and mixed with homologous antibody, the particles aggregate quantitatively and specifically. The maximum antibody titers, however, fall within a narrow range of Ag concentration, within which the effects of FAG are optimized. Excess FAG competes with BAG for antibodies in serum thus decreasing the antibody titer, whereas insufficient FAG results in a decrease of particle stability, probably due to the lowering of the zeta potential (8). To achieve optimum test reactivity, it is important to establish the most favorable ratio of FAG to BAG in the suspension.

This report describes the optimal distribution of Ag in a model latex test suspension. It also confirms that BAG is essential to particle aggregation, whereas FAG in appropriate ratios ensures both the stability of the suspension and the sensitivity of the test.

MATERIALS AND METHODS

Antigen and antisera. *E. coli* 0111:B4 was grown at 37°C for 16 h in beef heart infusion-1% glucose broth with or without 10 μ Ci of [³²P]orthophosphoric acid (New England Nuclear Corp.) per ml of medium. Ag was obtained from the bacteria by ethylenediaminetetraacetate extraction (6) and lyophilized. Antisera against *E. coli* 0111:B4 cells were prepared as previously described (5).

Latex preparations for serological test. (i) FAG-

BAG suspensions. Portions (0.1 ml) of 0.81- μ m Difco latex stock suspension were mixed with 0.5 ml of Ag at different concentrations and gently rotated for 30 min. Each volume was then brought to 10 ml with glycine-buffered saline (GBS), pH 8.2.

(ii) **BAG suspensions.** A 5-ml volume of each FAG-BAG suspension was centrifuged at 30,000 $\times g$ for 30 min at 5°C. The sediment was washed once with GBS and then suspended in 0.05 ml of latex supernatant (4), obtained by centrifugation of latex stock suspension, to restore the smooth, even suspension. The volume was brought to 5 ml with GBS.

(iii) **Latex suspensions in GBS and bovine serum albumin.** Portions (0.1 ml) of latex stock suspension were mixed with 9.9 ml of either GBS (latex-GBS) or GBS containing 0.1% bovine serum albumin, type F (Sigma Chemical Co.) (latex-0.1% BAF).

Latex preparation for study of Ag distribution in latex reagent. Batches (22 ml) of FAG-BAG latex suspension, containing variable added amounts of labeled Ag, were prepared as described above. For each suspension, 1 ml was used to count the number of latex particles and 10 ml to count total radioactivity. To the remaining 11 ml, 10 mg of BAF was added to assist the formation of a compact pellet, and this mixture was centrifuged at 11,000 $\times g$ for 20 min at 25°C. The pellet was suspended in 11 ml of GBS-0.1% BAF containing unlabeled Ag to minimize labeled Ag desorption from the particles and was recentrifuged as above. The concentration of unlabeled Ag in each batch was the same as that used in the original preparation. The final pellet was suspended in 1.1 ml of GBS; portions of this suspension were used to determine the number of particles recovered (0.1 ml) and the radioactivity adsorbed on latex (1.0 ml).

Calculation of distribution of Ag in latex suspension. After the particles were centrifuged and washed, the radioactivities of the pellet, supernatant, and wash were counted. The counts of the latter two were combined, and the count per particle in the pellet was calculated. From this value and from a determination of the number of particles remaining in the supernatant and wash, the count due to these particles was calculated. The combined radioactivity of the pellet and these particles corresponded to BAG. The remaining count in the combined supernatant and wash corresponded to FAG.

Serological test. The course of latex agglutination was studied by two approaches: (i) adding preincubated Ag-antiserum mixture to both uncoated (latex-GBS) and coated (latex-0.1% BAF) latex particles, and (ii) adding antiserum to both BAg and FAg-BAg suspensions. In the first approach, Ag solutions were prepared at the same concentrations as the FAg-BAg suspensions. Two batches of antiserum dilutions were mixed with equal volumes of each Ag solution, incubated for 1 h at 37°C, and then mixed with 0.4 ml of either latex-GBS or latex-0.1% BAF. In the second approach, two batches of antiserum dilutions were mixed with equal volumes of each BAg and each FAg-BAg suspension.

These four reaction mixtures were then incubated for 2 h at 56°C, left overnight at 5°C, and centrifuged at $700 \times g$ for 3 min. The tests were read macroscopically, and each titer was recorded as the reciprocal of the highest serum dilution exhibiting 2+ agglutination.

Controls. Three controls were included with each radiometric determination. (i) The first control was adherence of Ag to glass. A solution of 100 μg of Ag in 10 ml of GBS in a Correx tube (Corning) was centrifuged at $11,000 \times g$ for 20 min at 25°C. The supernatant was decanted, the test tube was washed twice with 10 ml of GBS-0.1% BAF, and the radioactivity in the supernatant and wash fluids was determined.

(ii) Desorption of Ag from latex comprised the second control. Latex-Ag reagents containing 10, 50, and 100 μg of Ag per 10 ml, prepared as described above, were centrifuged and washed twice, and the radioactivity in the supernatant and wash fluids was determined.

(iii) Quenching by latex particles in aqueous and Aquasol media comprised the third control. Duplicate batches of various volumes of latex particles, from 0.02 to 0.4 ml, were added to 0.1-ml portions of a ^{32}P solution (156,943 cpm). One batch at each volume was brought to 10 ml with GBS-0.1% BAF. The other was brought to 1.0 ml with GBS-0.1% BAF and then mixed with 10 ml of Aquasol (New England Nuclear Corp.). The radioactivity in the suspensions was then measured.

Radiometric count. Each 1-ml sample of BAg was mixed with 10 ml of Aquasol, and the beta activity was measured by liquid scintillation. The beta activity in each 10-ml sample of FAg-BAg, supernatant, and wash was measured by the Cerenkov radiation (4). When counted in Aquasol, the initial specific activity was 1,490 cpm/ μg of Ag. The ratio of counts in Aquasol to counts in GBS was 3.94.

Latex particle count. Samples of FAg-BAg (1.0 ml) and BAg (0.1 ml) were diluted in membrane-filtered (Millipore Corp.) GBS and counted with a Coulter counter (Coulter Electronics).

RESULTS

Controls. Minimal amounts of Ag adhered to the glass surface (0.5%) or desorbed (1%) from the particles. Latex particles at all concentrations tested had a minimal effect (0.2 to 0.5%)

on the counting efficiency of ^{32}P in aqueous and Aquasol media.

Particle count. The mean count for 18 FAg-BAg suspensions was 1.65×10^8 particles per ml. The relative standard deviation was 11.3%. All further results of this study are expressed in terms of 1.65×10^8 particles per ml.

Distribution of Ag in latex suspension. FAg was present at all concentrations of added Ag (Fig. 1). At 3.21 μg of added Ag per ml, the particles became virtually saturated; 1.02 μg was latex bound, and 2.19 μg was free in solution. At $\leq 0.825 \mu\text{g}/\text{ml}$, BAg accumulation exceeded FAg. From 0.825 to 3.21 $\mu\text{g}/\text{ml}$, FAg accumulated to higher levels than BAg.

Serological reactivity of the latex preparation. The preincubated Ag-antiserum mixtures added to latex-GBS and latex-0.1% BAF did not agglutinate the particles. Agglutination to a maximum of 2+ was attained with BAg preparations; graded agglutination (4+ to 1+) and maximum sensitivity were achieved with FAg-BAg preparations (Table 1). At each added-Ag concentration, the extent of agglutination depended on the antibody concentration in the serum, and vice versa. Maximum serological reactivity was attained at a concentration beyond the saturation point of the particles and within a narrow range of added-Ag concentration (3.21 to 20.75 $\mu\text{g}/\text{ml}$) (Table 2).

Correlation of serological sensitivity and Ag distribution. Serological sensitivity was correlated with the distribution of FAg and BAg in the latex suspension, expressed as the ratio of FAg to BAg. The results (Table 2) indi-

FIG. 1. Distribution of Ag added to 1-ml FAg-BAg latex suspensions.

TABLE 1. Titration of anti-*E. coli* antiserum with suspensions of BAg and FAg-BAg

Ag prepn ^a	Ag added (μ g/ml) ^b	Serum dilution									
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024
BAg	0.5	±	±	±	±	±	±	±	±	±	— ^c
	1	—	—	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—	—	—
	3	—	—	—	—	—	—	—	—	—	—
	5	±	±	±	±	±	±	±	±	±	±
	10	+	+	+	2+	2+	2+	+	+	+	±
	20	+	2+	2+	2+	2+	2+	2+	2+	—	±
	50	2+	2+	2+	2+	2+	2+	2+	2+	—	±
	100	2+	2+	2+	2+	2+	2+	2+	2+	—	±
FAg-BAg	0.5	—	—	—	—	—	—	—	—	—	—
	1	±	—	—	—	—	—	—	—	—	—
	2	2+	2+	2+	2+	+	±	—	—	—	—
	3	2+	3+	3+	3+	2+	±	±	—	—	—
	5	3+	3+	3+	3+	2+	+	+	±	—	—
	10	3+	4+	4+	3+	3+	3+	2+	2+	2+	—
	20	4+	4+	4+	4+	3+	3+	3+	2+	2+	+
	50	4+	4+	4+	4+	3+	3+	2+	±	—	—
	100	4+	4+	4+	4+	3+	3+	2+	±	—	—

^a Neither preparation agglutinated with GBS.^b Amount of Ag added per milliliter of latex suspension.^c Negative result.

TABLE 2. Relationship between serological sensitivity and FAg:BAg ratio in four sera

Zone ^a	Ag added (μ g/ml)	FAg:BAg	Serum titer ^b			
			1	2	3	4
3	96.37	56	128	16	128	128
	50.49	34	256	32	256	256
2	20.75	14.80	512	64	512	1024
	10.02	6.30	512	128	512	1024
	3.21	2.10	32	256	1024	512
1	1.93	1.64	16	128	256	128
	1.27	1.59		128	128	
	0.664	0.90		64		
	0.33	0.74		8		

^a Results were grouped into three zones according to particle stability and serological sensitivity.^b Reciprocal of serum dilution exhibiting 2+ agglutination.

cate three zones of reactivity according to particle stability and serological sensitivity. In zone 1 (ratios of ≤ 1.64), there was little or no reactiv-

ity. Antigen was still being adsorbed on the particles (Fig. 1), which were unstable and tended to form aggregates that settled rapidly (Fig. 2B, C). These aggregates were readily suspended by shaking. At a ratio of 2.10 and above, the suspension was stabilized (Fig. 2D), and maximum particle adsorption of Ag was reached. In zone 2 (ratios of 2.10 to 14.80) optimum reactivity was attained. In zone 3 (ratios of ≥ 34) the particles remained stable, but the test sensitivity decreased.

DISCUSSION

In a previous report on the quantitative determination of *E. coli* antibody (5), we observed that the maximum test reactivity fell within a narrow range of Ag concentration. The present study was undertaken to investigate the relationship between the FAg:BAg ratio and the sensitivity of the test.

The need for adsorption of Ag to latex was demonstrated in this study by the lack of agglutination of the latex particles in GBS or 0.1%

FIG. 2. Stability of latex suspensions at different ratios of FAg to BAg. The latex is suspended in (A) GBS; (B and C) FAg-BAg suspensions in zone 1, with ratios of 1.02 (equivalent to 1 $\mu\text{g/ml}$) and 1.78 (2 $\mu\text{g/ml}$), respectively; (D) FAg-BAg suspension in zone 2, with a ratio of 6.3 (10 $\mu\text{g/ml}$). After 1-h incubation at 25°C, the particles were evenly suspended in tubes A and D; aggregation occurred to some extent in tube B and maximally in tube C.

BAF suspensions with the Ag-antibody complexes. This observation is at variance with findings of Donaldson and Pennington (2), who reported specific agglutination with unadsorbed Ag in an albumin-antialbumin system.

Aggregation was observed in our system when either BAg or FAg-BAg suspensions were mixed with homologous antibodies. Only in the FAg-BAg preparations, however, was there graded aggregation and, within a narrow range of added-Ag concentration (Zone 2), maximum serological reactivity. Reactivity was low or absent in zone 1 and low in zone 3.

At the threshold to zone 2, the particles stabilized. We postulated that after saturation the particles acquired lyophilic properties, thus becoming stable. Oreskes and Singer (8) and Singer et al. (10) have shown that the latex suspension, which is originally stable (Fig. 2) because of the negative charge around the particles and therefore behaves as a lyophobic colloid, aggregates in the presence of a low concentration of serum protein (10 $\mu\text{g/mg}$ of latex). They attributed this aggregation to the lowering of the zeta potential by the added protein. As still more protein was added, the particles in suspension became stable again, assuming the lyophilic properties of the protein. These authors concluded that stability is associated with the addition of sufficient protein to form an adsorbed monolayer on the latex particles.

Zone 2 is presumably optimum for Ag-antibody interaction and/or for lattice formation.

Oreskes and Singer (7) suggested that agglutination is due either to antibody interaction with Ag at the particle surface (destabilization) and/or to cross-linking of the reactants (Fig. 3). Since optimum serological reactivity was not attained in our system with BAg preparations, our results suggest that at appropriate concentrations of FAg, lattice formation predominates over any simultaneous latex destabilization.

In zone 3 the excess of FAg apparently reacts with antibody to form soluble FAg-antibody-FAg complexes, reducing the number of anti-

FIG. 3. Simplified models of three theoretical forms of Ag-antibody complex in latex agglutination. (A) Destabilization by interaction of BAg with antibody (Ab); (B) cross-linking of two BAg by Ab alone; (C) cross-linking of two BAg-Ab complexes and free Ab by FAg. Use of BAg alone would result in (A) and (B) aggregates. Use of FAg-BAg would result also in the larger (C) aggregates, the inclusion of which accounts for the improved sensitivity.

bodies free to react with BAg. Beyond a certain concentration, therefore, FAg inhibits particle aggregation and results in a lower measured antibody titer.

To summarize our findings: (i) For specific aggregation, Ag must be adsorbed on the latex particles before the serum is added. (ii) FAg is essential for maximum serological reactivity. It stabilizes the latex suspension and enhances the formation of lattices. (iii) A combination of BAg and FAg is essential for maximum sensitivity. (iv) For each test system, it is important to determine the optimum range of FAg:BAg ratios to achieve maximum reactivity.

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