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Vaccine 22 (2004) 1973–1984
Vaccine
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Distribution of adsorbed antigen in mono-valent and combination vaccines

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Received 13 August 2003; received in revised form 20 October 2003; accepted 23 October 2003

Abstract

The distribution of alpha-casein, bovine serum albumin (BSA), myoglobin and recombinant protective antigen (rPA) in mono-valent and combination vaccines containing aluminum hydroxide adjuvant was studied by fluorescence microscopy and flow cytometry. Green and red fluorescent probes were conjugated to the antigens. Adsorption isotherms of the fluorescently labeled proteins to aluminum hydroxide adjuvant demonstrated that incorporation of the fluorescent probe did not significantly affect the adsorption. In mono-valent vaccine systems, antigen adsorption occurred within one minute and uniform surface coverage of the adjuvant aggregates was observed within 1 h. Content uniformity was achieved through a cycle of de-aggregation and re-aggregation of the aluminum hydroxide adjuvant aggregates caused by mixing. For combination vaccines, two antigens were adsorbed separately to the aluminum hydroxide adjuvant prior to combination. Following combination, cycles of de-aggregation and re-aggregation occurred due to mixing, which led to uniform distribution of both antigens. The results of this study indicate that content uniformity should not be an issue during the production of mono-valent or combination vaccines as long as adequate mixing procedures are followed.

Keywords: Content uniformity in mono-valent and combination vaccines; Antigen adsorption by aluminum hydroxide adjuvant; Fluorescence microscopy; Flow cytometry

1. Introduction

Aluminum-containing adjuvants were discovered in 1926 by Glenny et al. [1]. They precipitated diphtheria toxoid with alum and injected the suspension into guinea pigs. The precipitated diphtheria toxoid provided greater protection to the guinea pigs than the toxoid alone. They also found that there was no diphtheria toxoid in the supernatant of the precipitate. This led to the conclusion that adsorption of the antigen was critical for enhanced immunogenicity. For this reason an important goal in the formulation and manufacture of vaccines with aluminum-containing adjuvants is adsorption of the antigen to the adjuvant. It is important to assess the content uniformity in such vaccines as the dose of most antigens is in micrograms while the quantity of aluminum-containing adjuvant may be up to 0.85 mg Al/dose [2]. In this study, the adsorption of antigen to aluminum hydroxide adjuvant was monitored during production of mono-valent vaccines by conjugating a green fluorescent dye to the antigen and detecting the antigen by fluorescence microscopy and flow cytometry. A solution of the antigen was either added to all of the aluminum hydroxide adjuvant with mixing or a solution of the antigen was added to a portion (25%) of the aluminum hydroxide adjuvant and the remainder of the aluminum hydroxide adjuvant was added after a 1 h mixing period.

Content uniformity in combination vaccines was studied by conjugating one antigen to a green fluorescent dye while the other antigen was conjugated to a red fluorescent dye. Each antigen was adsorbed to aluminum hydroxide adjuvant prior to combination. Once the mono-valent vaccines were combined, the distribution of the antigens was monitored by fluorescence microscopy.

Aluminum hydroxide adjuvant has a fibrous morphology with a primary particle size of 4.5 nm × 2.2 nm × 10 nm [3]. However, the primary particles do not exist as solitary particles but form loose aggregates in the size range of 1–10 μm [4]. Aluminum hydroxide adjuvant is chemically aluminum

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doi:10.1016/j.vaccine.2003.10.040

oxyhydroxide [5] and therefore contains hydroxyl groups that can either accept or donate protons. The isoelectric point of aluminum hydroxide adjuvant is 11.4 [4].

The surface hydroxyls of aluminum hydroxide adjuvant provide the basis for the major mechanisms of adsorption of antigens: electrostatic adsorption when the antigen and adjuvant have opposite charges and ligand exchange when the antigen contains a phosphate group that is able to exchange with a hydroxyl group at the surface of the adjuvant. Of these adsorption mechanisms, electrostatic attraction is the most frequently encountered while ligand exchange produces the strongest adsorption [6].

Antigens were selected for this study that adsorbed by strong electrostatic attraction, weak electrostatic attraction and ligand exchange. Bovine serum albumin (BSA) has a molecular weight of 66,000 and an isoelectric point of 5.0 [7]. Protective antigen is a component of the toxin secreted by *Bacillus anthracis*. Recombinant protective antigen (rPA) has a molecular weight of 83,000 and an isoelectric point of 5.6 [8]. Both BSA and rPA are free of phosphate and adsorb to aluminum hydroxide adjuvant at pH 7.4 by strong electrostatic attraction [8,9]. Myoglobin has a molecular weight of 16,000, an isoelectric point of 7.2 and contains no phosphate [7]. Myoglobin adsorbs to aluminum hydroxide adjuvant by weak electrostatic attraction at pH 7.4 [9]. Alpha-casein has a molecular weight of 26,000, an isoelectric point of 4.6 and contains eight phosphate groups [10,11]. The presence of the phosphate groups causes alpha-casein to adsorb to aluminum hydroxide adjuvant by the ligand exchange mechanism [12].

2. Materials and methods

2.1. Materials

Bovine serum albumin, myoglobin and alpha-casein were obtained commercially (Sigma, St. Louis, MO). Stock rPA solutions containing 2 mg rPA/ml in Dulbeccos phosphate buffered saline (DPBS) at pH 7.4 or 8.3 were donated by Dr. Scott Jendrek (SAIC-Frederick Inc., National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD).

2.2. Fluorescent labeling of antigens

Bovine serum albumin was labeled with either BODIPY FL, a green fluorescent dye, or BODIPY TR, a red fluorescent dye (Molecular Probes, Eugene, OR). A 5 mg/ml solution of BSA was prepared by dissolving 75 mg of BSA in 14 ml of doubly distilled water (ddH_2O), adjusting the pH to 8.3 with 0.1 M NaOH and adjusting the volume to 15 ml with ddH_2O. The pH of the BSA solution was adjusted to 8.3 because the reaction is most efficient between pH 7.5 and 8.5 [13] and addition of the fluorescent dye will slightly lower the pH. For the labeling reaction, 1 ml of the BSA solution was combined with either 25 μl of 5 mg/ml BODIPY FL or 36 μl of 5 mg/ml BODIPY TR and mixed using a vortex mixer for 1 h at room temperature. Following mixing, the labeled BSA was separated from unreacted dye by size exclusion chromatography using Sephadex G-50 fine (Sigma, St. Louis, MO) equilibrated with ddH_2O. The fluorescent dye labeled BSA eluted from the column first and was collected. The concentration of the labeled BSA was determined by the BCA assay (Pierce, Rockford, IL).

Recombinant protective antigen was labeled with BODIPY FL. For the labeling reaction, 1 ml of the rPA stock solution in DPBS at pH 8.3 was combined with 10 μl of 5 mg BODIPY FL/ml and mixed using a vortex mixer for 1 h at room temperature. The labeled rPA was separated from unreacted dye by size exclusion chromatography using Sephadex G-50 fine equilibrated with phosphate buffered saline (PBS) at pH 7.4.

Samples of myoglobin were labeled with either BODIPY FL or BODIPY TR. A 2 mg/ml solution of myoglobin was prepared by dissolving 30 mg of myoglobin in 14 ml of ddH_2O, adjusting the pH to 8.3 with 0.1 M NaOH and adjusting the volume to 15 ml with ddH_2O. For the labeling reaction, 1 ml of the myoglobin solution was combined with either 26 μl of 5 mg/ml BODIPY FL or 37 μl of 5 mg/ml BODIPY TR and mixed using a vortex mixer for 1 h at room temperature. The labeled myoglobin was separated from untreated dye as described for BSA.

Alpha-casein was labeled with either BODIPY FL or BODIPY TR. A 5 mg/ml solution of alpha-casein was prepared by dissolving 75 mg of alpha-casein in 14 ml of ddH_2O, adjusting the pH to 8.3 with 0.1 M NaOH and adjusting the volume to 15 ml with ddH_2O. For labeling, 1 ml of the alpha-casein solution was combined with either 68 μl of 5 mg/ml BODIPY FL or 98 μl of 5 mg/ml BODIPY TR and mixed using a vortex mixer for 1 h at room temperature. The labeled alpha-casein was separated from untreated dye as described for BSA.

2.3. Adsorption isotherms

The adsorption isotherms of both unlabeled and labeled BSA, myoglobin and alpha-casein were determined by combining 100 μl of aluminum hydroxide adjuvant (Rehydragel HPA, Reheis, Berkeley Heights, NJ) with 100 μl of the appropriate protein solution. The commercial adjuvant was diluted to 0.1 mg Al/ml with 0.01 M veronal buffer (Sigma, St. Louis, MO) and adjusted to pH 7.4 with 0.1 M NaOH or 0.1 M HCl. The concentration ranges of the protein solutions, adjusted to pH 7.4 with 0.1 M NaOH or 0.1 M HCl, were: BSA, 200–400 μg/ml; myoglobin, 75–200 μg/ml; and alpha-casein, 175–300 μg/ml. The adjuvant and protein mixtures were mixed using a vortex mixer at room temperature for 1 h. Preliminary experiments indicated that equilibrium was reached in less than 30 min. The mixtures were then centrifuged and the protein concentration of the clear supernatant determined by the BCA assay. The adsorptive

capacity was calculated from the linearized form of the Langmuir equation as recently described [8]. The reported adsorptive capacity was the mean of three separate adsorption isotherms. The 95% confidence interval was also calculated (SAS, version 8.2, SAS Institute, Cary, NC) using the three separate adsorption isotherms.

The adsorption isotherms of unlabeled and labeled rPA were determined using a 200 μg Al/ml suspension of aluminum hydroxide adjuvant (Alhydrogel 2.0%, HCI Biosector, Frederikssund, Denmark). The commercial adjuvant was diluted with PBS. The rPA stock solution in DPBS at pH 7.4 was diluted with PBS to produce rPA solutions ranging from 40 to 175 μg/ml. Solutions of BODIPY FL-labeled rPA were prepared in the same concentration range by dilution with PBS. Adsorption isotherms were prepared by mixing 100 μl of diluted adjuvant with 100 μl of the appropriate rPA solution.

2.4. Distribution of antigen in mono-valent vaccines

To observe the distribution of BSA, myoglobin and alpha-casein, 10 μg/ml solutions of each of the BODIPY FL-labeled proteins were prepared by dilution with 0.01 M veronal buffer at pH 7.4. A 100 μg Al/ml stock suspension of aluminum hydroxide adjuvant was also prepared by dilution with 0.01 M veronal buffer at pH 7.4. Prior to combination, images of the aluminum hydroxide adjuvant without protein were obtained at 40× magnification in phase contrast mode. Five milliliters of protein solution was then combined with 5 ml of adjuvant suspension and mixed with a magnetic stir bar. For observation by fluorescence microscopy, 5 μl samples were taken after 1, 15, 30, 45 and 60 min of mixing. Images of the samples were obtained immediately at 40× magnification in both fluorescence and phase contrast modes. The fluorescence and phase contrast images were overlaid and cropped with Adobe Photoshop 7.0(r) (Adobe, San Jose, CA). For analysis by flow cytometry, 500 μl samples were taken after 1, 5, 10, 15, 20, 30, 45 and 60 min of mixing. Each sample was assayed immediately by flow cytometry (Epics XL, Coulter, Fullerton, CA). A minimum of 10,000 events were collected and the data was analyzed with WIN MDI(r) (Joe Trotter, Scripps Institute, La Jolla, CA) to determine the average fluorescence intensity and the percentage of particles with protein adsorbed. To obtain the percentage of particles with protein adsorbed, aluminum hydroxide adjuvant was first assayed by flow cytometry for fluorescence. On the fluorescence intensity histogram it was determined that 99% of the adjuvant aggregates had a fluorescence intensity less than 6. Therefore, when the model vaccines were assayed any adjuvant aggregate with a fluorescence intensity greater than 6 was considered to have antigen adsorbed.

To observe the distribution of rPA, a 20 μg/ml solution of labeled rPA was prepared by dilution with PBS at pH 7.4. A 290 μg Al/ml suspension of aluminum hydroxide adjuvant was prepared by dilution with PBS at pH 7.4. The procedure described above was followed for examination by fluorescence microscopy and flow cytometry except the magnification was 60× rather than 40×.

Fluorescence microscopy was used to observe the distribution of BSA, myoglobin and alpha-casein adsorbed to aluminum hydroxide adjuvant when additional adjuvant was added to the system. Solutions (10 μg/ml) of each of the BODIPY FL-labeled proteins were prepared by dilution with 0.01 M veronal buffer at pH 7.4. A 100 μg Al/ml aluminum hydroxide adjuvant suspension (Rehydragel HPA, Reheis, Berkeley Heights, NJ) was prepared by dilution with 0.01 M veronal buffer at pH 7.4. Two milliliters of protein solution was combined with 2 ml of adjuvant suspension and mixed using a magnetic stir bar for 1 h. Following mixing, a 5 μl sample was taken and images were obtained at 40× magnification in fluorescence and phase contrast modes. Next, 6 ml of adjuvant suspension was added. Five microliter samples were taken after 1, 15, 30, 45 and 60 min of mixing. Images of each of the samples were obtained immediately at 40× magnification in fluorescence and phase contrast modes. The fluorescence and phase contrast images obtained were overlaid and cropped as described above.

2.5. Distribution of antigen in combination vaccines

The distribution of antigen during the production of combination vaccines was observed using fluorescence microscopy. For each combination, one protein was labeled with BODIPY FL (green) and the other protein was labeled with BODIPY TR (red). Mono-valent vaccines were prepared by combining 5 ml of 10 μg/ml protein solution with 5 ml of 100 μg Al/ml aluminum hydroxide adjuvant (Rehydragel HPA, Reheis, Berkeley Heights, NJ) and mixing for 1 h using a magnetic stir bar. Following mixing, 5 μl samples of each of the mono-valent vaccines were taken and images were obtained at 40× magnification in fluorescence and phase contrast modes. Five milliliters of each of the mono-valent vaccines were combined and mixed with a magnetic stir bar. Samples were taken after 1, 15, 30, 45 and 60 min of mixing. Images were obtained immediately at 40× magnification in fluorescence and phase contrast modes. The fluorescence and phase contrast images were overlaid and cropped as described previously.

3. Results

3.1. Adsorption isotherms

The adsorption isotherms of the unlabeled and labeled proteins by aluminum hydroxide adjuvant were obtained to determine if the fluorescent label affects adsorption. The mean adsorptive capacities from three separate adsorption isotherms and the 95% confidence interval for each protein are presented in Table 1. The labeling of each protein did

Table 1
Mean adsorptive capacities from three separate adsorption isotherms

Protein	Mean adsorptive capacity (mg/mg Al)	95% confidence interval (±mg/mg Al)
BSA	2.5	0.15
BSA-FL	2.6	0.55
BSA-TR	2.7	0.13
AC[a]	2.1	0.73
AC-FL	2.1	0.03
AC-TR	1.7	0.43
Myo[b]	1.2	0.22
Myo-FL	1.2	0.67
Myo-TR	1.2	0.43
rPA	0.23	0.10
rPA-FL	0.27	0.07

[a] Alpha-casein.
[b] Myoglobin.

not produce a statistically significant change in the adsorptive capacity. Thus, studies with the labeled proteins can be applied to the unlabeled proteins.

3.2. Distribution of antigen in mono-valent vaccines

The concentration of antigen in most commercial vaccines is well below the adsorptive capacity of aluminum hydroxide adjuvant. Therefore, the distribution studies were performed using model vaccines containing 100 μg antigen/mg Al for BSA, alpha-casein and myoglobin. The rPA model vaccine contained 69 μg rPA/mgAl. As seen in Table 1, the concentrations of antigens used in the distribution studies were below the adsorptive capacity of each antigen.

Although aluminum hydroxide adjuvant is composed of very small primary particles [3], the primary particles form irregularly shaped aggregates having diameters between 5 and 10 μm. Four representative aluminum hydroxide adjuvant aggregates are shown in Fig. 1.

When a solution of BODIPY FL-labeled BSA was mixed with aluminum hydroxide adjuvant, each adjuvant aggregate exhibited a uniform green fluorescence within 1 min of mixing (Fig. 2). This is visual evidence that BSA adsorbs quickly to aluminum hydroxide adjuvant. The adjuvant aggregates continue to exhibit a uniform green fluorescence during 60 min of mixing.

Aluminum hydroxide adjuvant was analyzed by flow cytometry and 99% of the adjuvant aggregates had a fluorescence intensity less than six. Therefore, in the mono-valent vaccines, any adjuvant aggregate with a fluorescence intensity greater than 6 was considered to have BSA adsorbed. Analysis of the BSA mono-valent vaccine by flow cytometry after 1 min of mixing (Fig. 3) indicated that 99% of the adjuvant aggregates had BSA adsorbed.

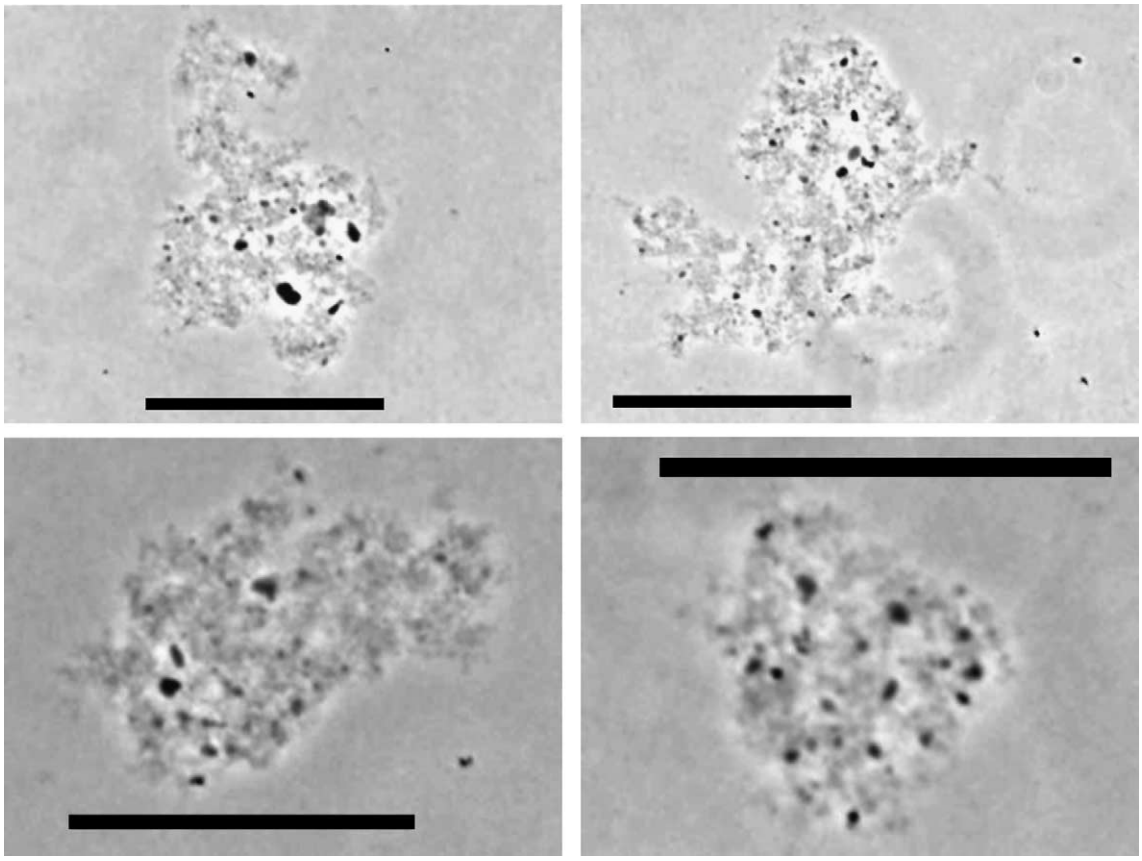

Fig. 1. Phase contrast images of four aggregates of aluminum hydroxide adjuvant. The bars represent 5 μm.

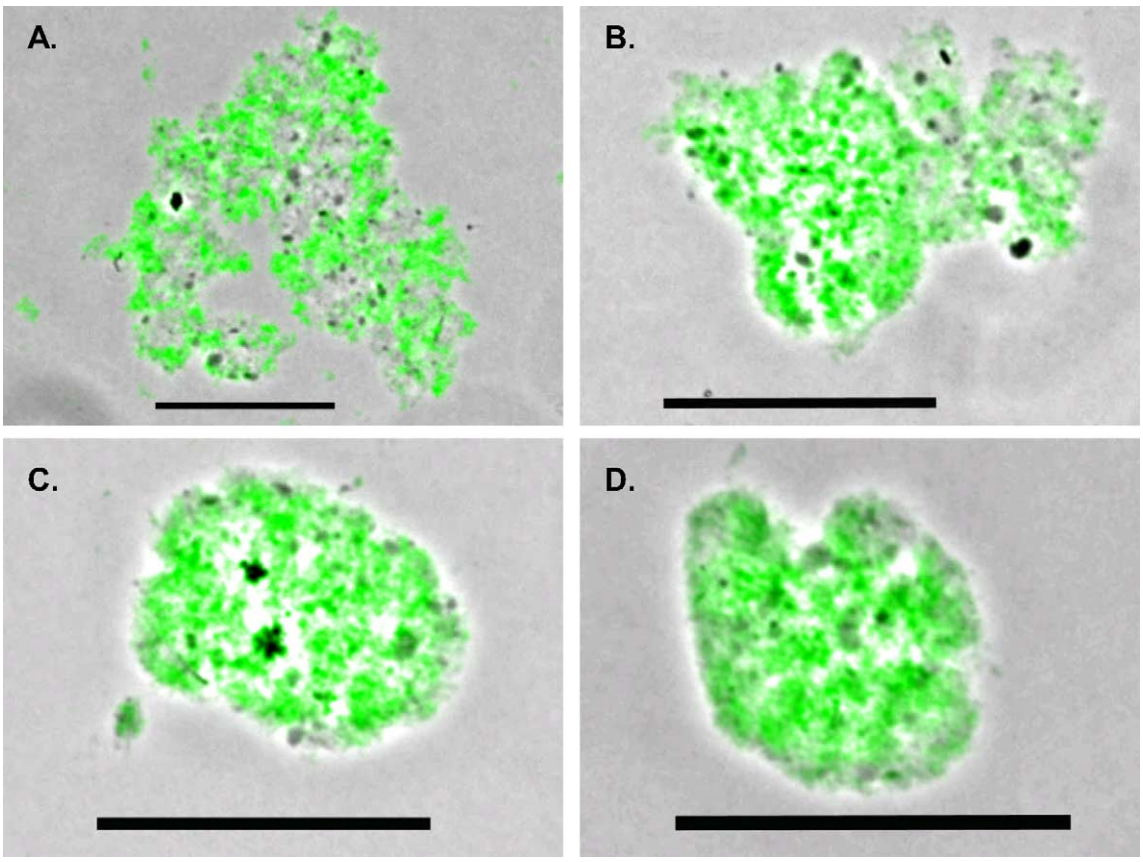

Fig. 2. Adsorption of bovine serum albumin labeled with BODIPY FL to aluminum hydroxide adjuvant: (A) 1 min of mixing; (B) 30 min of mixing; (C) 45 min of mixing; (D) 60 min of mixing. The bars represent 5 μm.

The adsorption of BODIPY FL-labeled rPA by aluminum hydroxide adjuvant was monitored by fluorescence microscopy. Adsorption occurred rapidly and the adjuvant aggregates exhibited a uniform green fluorescence at all time points similar to that seen in the BSA model vaccine (Fig. 2). Examination by flow cytometry after 1 min of mixing indicated that 99% of the adjuvant aggregates had rPA adsorbed. Thus, the two antigens that adsorb by strong electrostatic attraction, BSA and rPA, exhibited rapid and uniform adsorption by aluminum hydroxide adjuvant.

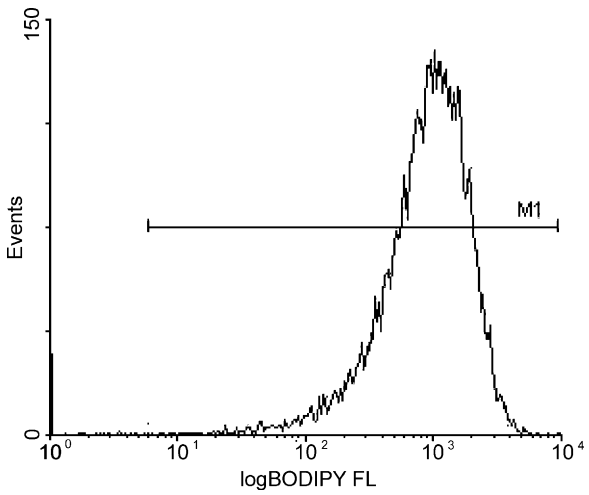

Fig. 3. Fluorescence intensity histogram for a mixture of bovine serum albumin labeled with BODIPY FL and aluminum hydroxide adjuvant after 1 min of mixing. Adjuvant aggregates lying in the M1 region were considered to have BSA adsorbed.

Myoglobin exhibits a small negative surface charge at pH 7.4 and therefore is adsorbed by weak electrostatic attraction. As seen in Fig. 4, the adsorption of myoglobin did not occur as rapidly and uniformly as observed for BSA or rPA. The fluorescent image taken after 1 min of mixing was typical of many adjuvant aggregates photographed. Regions of the adjuvant aggregate exhibited green fluorescence after 1 min of mixing but the green fluorescence was not uniformly distributed over the adjuvant aggregate. The grey regions indicate areas where no myoglobin was adsorbed. The protein was more completely distributed after 30 min of mixing but areas without adsorbed myoglobin were still apparent. The adjuvant aggregates finally exhibited a uniform green fluorescence after 60 min of mixing. Analysis by flow cytometry after 1 min of mixing indicated that 99% of the adjuvant aggregates had myoglobin adsorbed. When the fluorescent photomicrographs of myoglobin mixed with aluminum hydroxide adjuvant (Fig. 4) are compared to those of BSA (Fig. 2) and rPA, it is clear that antigens that adsorb

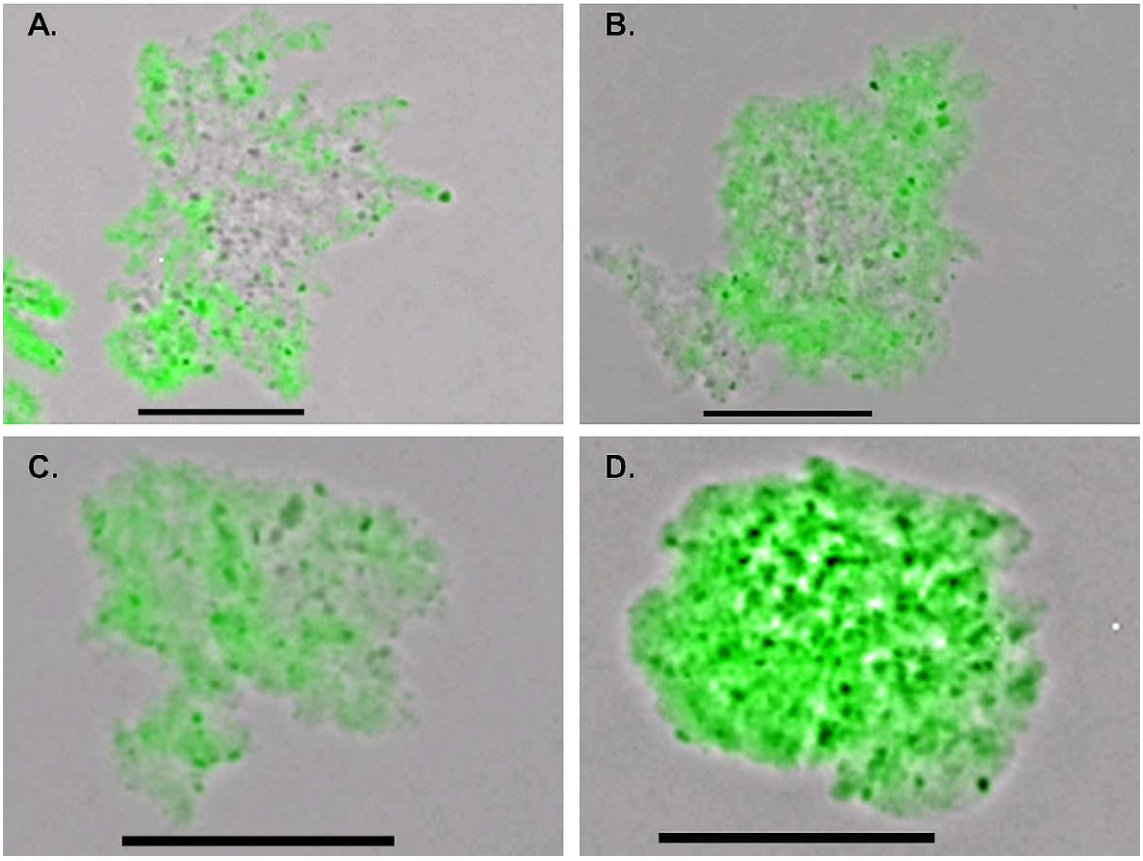

Fig. 4. Adsorption of myoglobin labeled with BODIPY FL to aluminum hydroxide adjuvant: (A) 1 min of mixing; (B) 15 min of mixing; (C) 30 min of mixing; (D) 60 min of mixing. The bars represent 5 μm.

by weak attractive forces require a longer mixing time for uniform distribution. In spite of the weak electrostatic attraction, myoglobin exhibited uniform distribution after 60 min of mixing.

Alpha-casein is adsorbed by aluminum hydroxide adjuvant by ligand exchange. This adsorption mechanism is so strong that alpha-casein does not elute from aluminum hydroxide adjuvant upon exposure to interstitial fluid [14]. When BODIPY FL-labeled alpha-casein was combined with aluminum hydroxide adjuvant, adsorption occurred rapidly and uniform coverage of the adjuvant aggregate was observed after 1 min of mixing (Fig. 5). Flow cytometry revealed that 99% of the adjuvant aggregates had alpha-casein adsorbed after one minute of mixing. The strong attractive force of ligand exchange is believed to be responsible for the rapid and complete adsorption of alpha-casein by aluminum hydroxide adjuvant.

Mono-valent vaccines may also be produced by adsorbing the antigen to a portion of the aluminum-containing adjuvant and subsequently adding the remaining adjuvant. Fig. 6A shows an adjuvant aggregate following mixing with BODIPY FL-labeled BSA for 1 h. One minute after additional aluminum hydroxide adjuvant was added with mixing, the adjuvant aggregates were composed of large green fluorescent regions with BSA adsorbed and large grey regions free of BSA (Fig. 6B). Continued mixing caused the well-defined regions of fluorescence to become smaller and more highly dispersed throughout the adjuvant aggregate (Fig. 6B–F). This suggests that mixing caused the adjuvant aggregates to de-aggregate and then to re-aggregate by combining BSA-adsorbed aggregate fragments with aggregate fragments that were free of BSA. This cycle of de-aggregation and re-aggregation led to uniform distribution of the BSA throughout the vaccine. It is likely that this mechanism for achieving content uniformity also occurred in the vaccines produced by mixing a solution of antigen with the entire aluminum hydroxide adjuvant (Figs. 2, 4 and 5).

A myoglobin vaccine was prepared by adsorbing BODIPY FL-labeled myoglobin to 25% of the aluminum hydroxide adjuvant in the formulation and subsequently adding the remaining 75% of the adjuvant. Fig. 7 indicates that content uniformity was achieved through mixing by the same de-aggregation and re-aggregation mechanism noted for BSA (Fig. 6). However, in the case of myoglobin, a second mechanism appears to be operating because the adjuvant aggregates observed at 15, 30 and 45 min appeared to be composed of strongly green fluorescent regions, similar in intensity to the adjuvant aggregate seen in Fig. 7A and weakly green fluorescent regions. The weak electrostatic

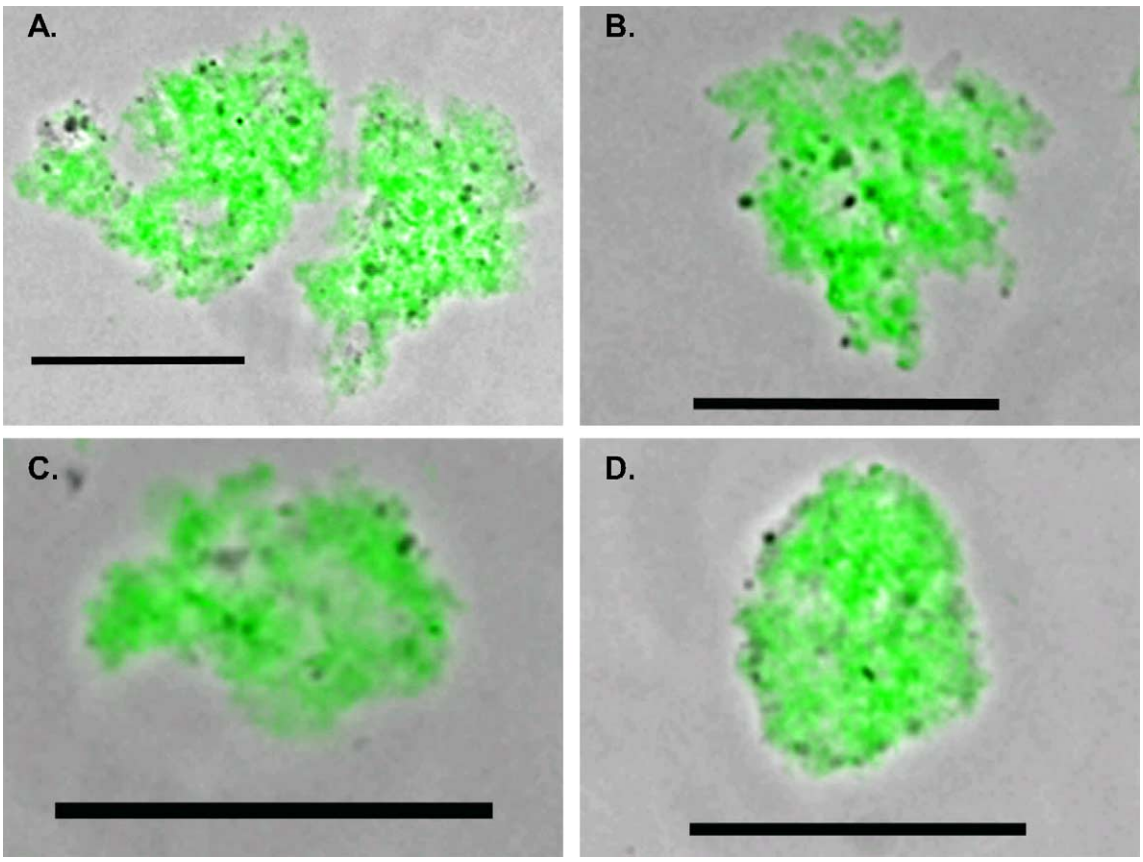

Fig. 5. Adsorption of alpha-casein labeled with BODIPY FL to aluminum hydroxide adjuvant: (A) 1 min of mixing; (B) 30 min of mixing; (C) 45 min of mixing; (D) 60 min of mixing. The bars represent 5 μm.

attraction between myoglobin and aluminum hydroxide causes myoglobin to elute from aluminum hydroxide adjuvant upon dilution with water [9]. The addition of 75% of the adjuvant following adsorption of the myoglobin to 25% of the adjuvant is equivalent to dilution with water. Thus, some myoglobin eluted and was re-adsorbed by the added myoglobin-free adjuvant. This elution and re-adsorption mechanism appears to be responsible for the weakly green fluorescent regions of the adjuvant aggregates seen in Fig. 7 while the de-aggregation and re-aggregation mechanism is responsible for the strongly green fluorescent regions seen in Fig. 7.

The distribution of BODIPY FL-labeled alpha-casein when the vaccine was prepared by adsorbing the alpha-casein to 25% of the aluminum hydroxide adjuvant and diluting with the balance of the adjuvant appears to occur by the same de-aggregation and re-aggregation mechanism observed for BSA. As seen in Fig. 8, progressively smaller dark green fluorescent and grey regions were seen in the adjuvant aggregates over the 60 min mixing period. Thus, antigens that adsorb to aluminum-containing adjuvants by strong electrostatic attraction, like BSA, or ligand exchange, like alpha-casein, were uniformly distributed through the vaccine by the de-aggregation and re-aggregation mechanism caused by mixing. Antigens that were adsorbed by weak electrostatic attraction, such as myoglobin, were distributed throughout the vaccine by both the de-aggregation and re-aggregation mechanism as well as elution of antigen due to dilution and re-adsorption. In either case, content uniformity was achieved as long as adequate mixing procedures were followed.

3.3. Distribution of antigens in combination vaccines

The distribution of antigens following the combination of two mono-valent vaccines was studied by labeling one antigen with a green fluorescent dye (BODIPY FL) and the other with a red fluorescent dye (BODIPY TR). The first system studied was green-labeled BSA adsorbed to aluminum hydroxide adjuvant combined with red-labeled BSA adsorbed to aluminum hydroxide adjuvant. This models combination vaccines in which both antigens are adsorbed by strong electrostatic attraction. The green fluorescent BSA adsorbed to aluminum hydroxide adjuvant and the red fluorescent BSA adsorbed to aluminum hydroxide adjuvant are shown in Fig. 9A and B. Following combination with mixing, the aggregates were composed of green fluorescent regions and red fluorescent regions. The large,

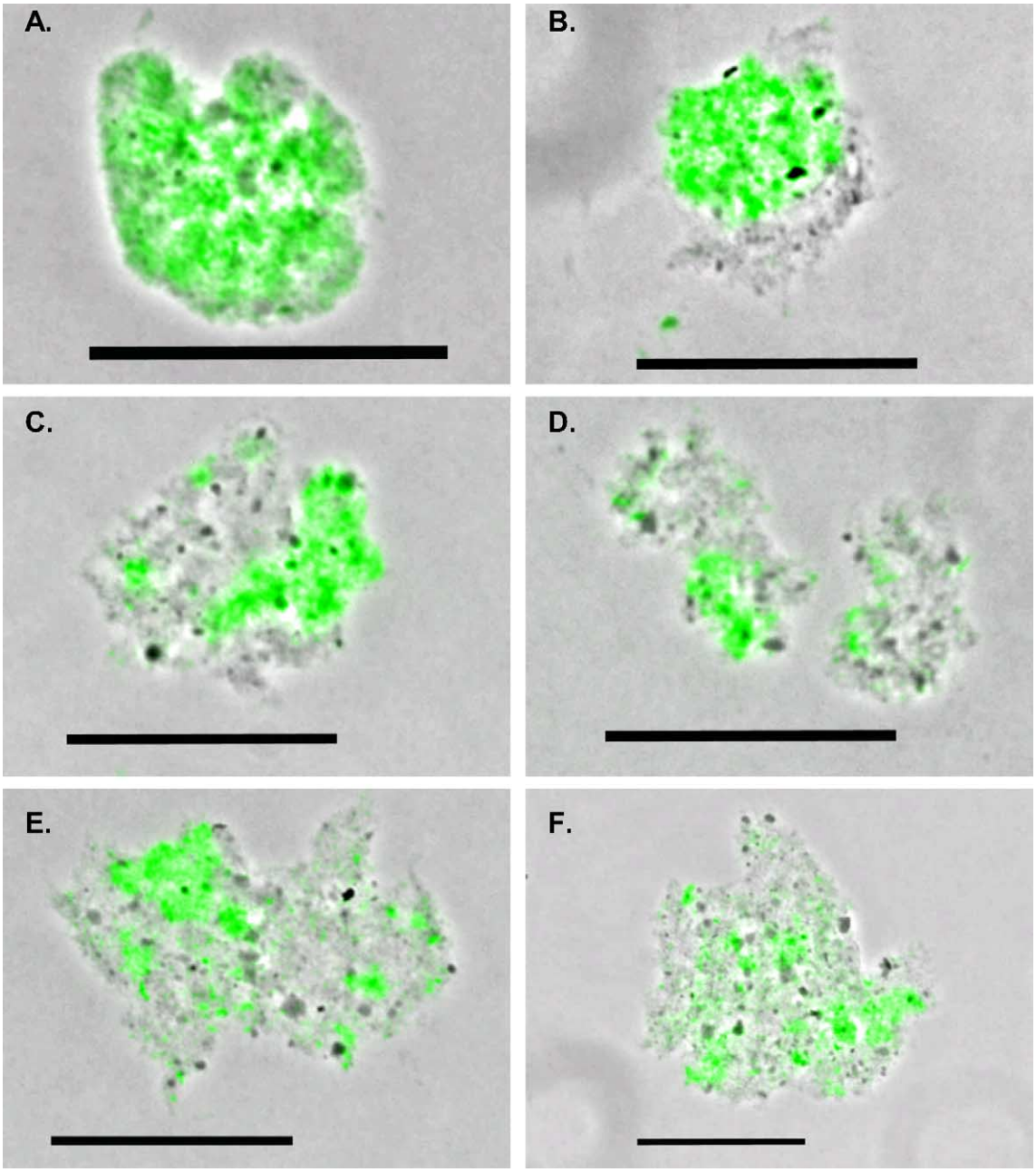

Fig. 6. Distribution of BODIPY FL-labeled bovine serum albumin in aluminum hydroxide adjuvant when the antigen was adsorbed to 25% of the adjuvant and the mixture diluted with the remaining 75% of the aluminum hydroxide adjuvant: (A) initial adsorption of BSA to aluminum hydroxide adjuvant; (B) 1 min after addition of balance of aluminum hydroxide adjuvant to A with mixing; (C) 15 min of mixing; (D) 30 min of mixing; (E) 45 min of mixing; (F) 60 min of mixing. The bars represent 5 μm.

well-defined fluorescent regions became smaller during the 60 min of mixing. The yellow color seen in Fig. 9C–F was caused by the three-dimensional aspect of the adjuvant aggregates that allowed green fluorescent and red fluorescent regions to be superimposed in some parts of the aggregate. A yellow color was generated by the computer program when the green and red fluorescence images were overlaid. This behavior is consistent with the de-aggregation and re-aggregation mechanism that was proposed in the discussion of the preparation of mono-valent vaccines. As with mono-valent vaccines, this mechanism produced uniform distribution of the antigens throughout the aluminum hydroxide adjuvant as long as adequate mixing was used.

The de-aggregation and re-aggregation mechanism also produced uniform distribution of antigens in combination vaccines composed of: two weakly adsorbed antigens (myoglobin/myoglobin); two antigens adsorbed by ligand

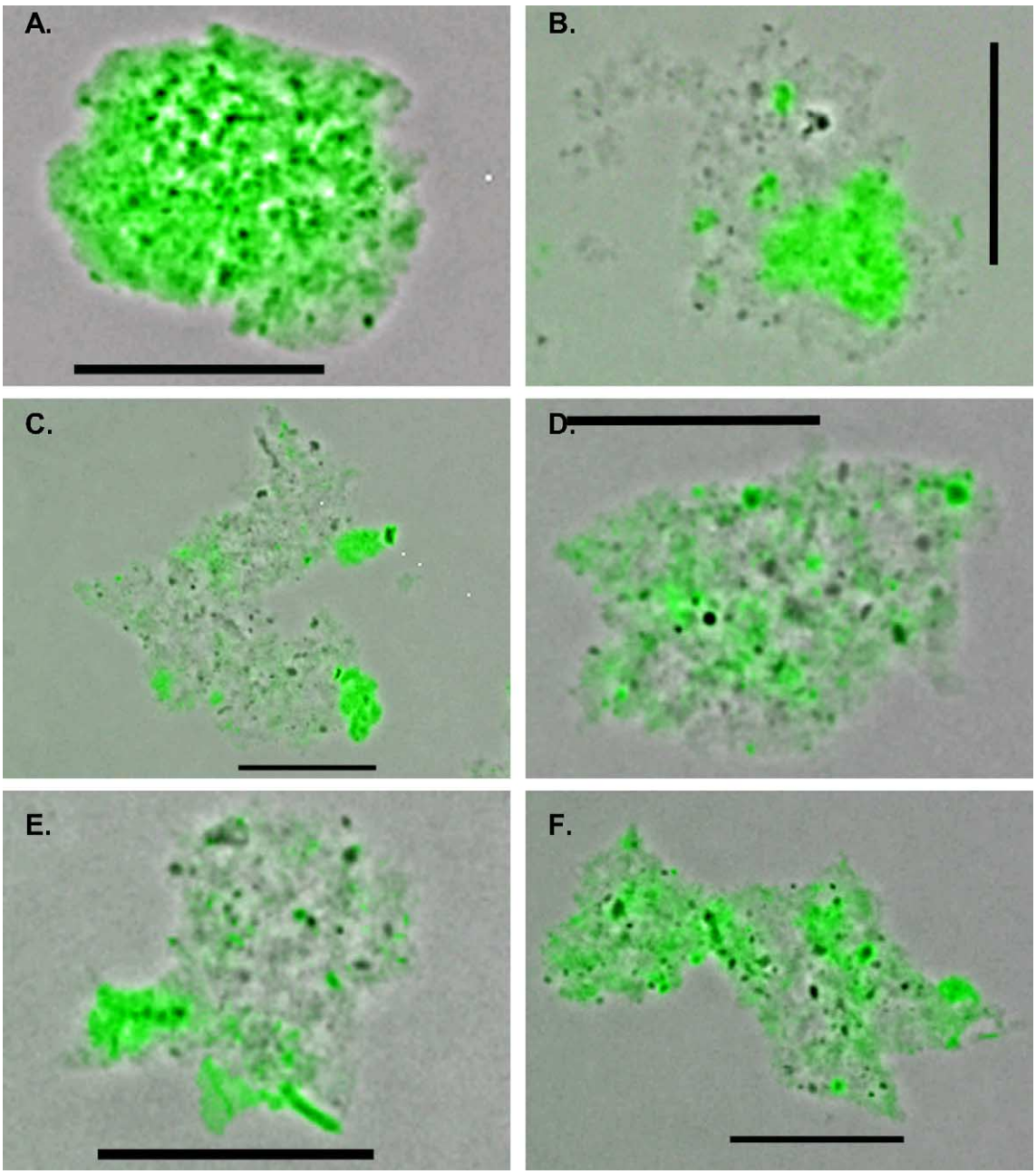

Fig. 7. Distribution of BODIPY FL-labeled myoglobin in aluminum hydroxide adjuvant when the antigen was adsorbed to 25% of the adjuvant and the mixture diluted with the remaining 75% of the aluminum hydroxide adjuvant: (A) initial adsorption of myoglobin to aluminum hydroxide adjuvant; (B) 1 min after addition of balance of aluminum hydroxide adjuvant to A with mixing; (C) 15 min of mixing; (D) 30 min of mixing; (E) 45 min of mixing; (F) 60 min of mixing. The bars represent 5 μm.

exchange (alpha-casein/alpha-casein); one antigen adsorbed by strong electrostatic attraction and one antigen adsorbed by weak electrostatic attraction (BSA/myoglobin); one antigen adsorbed by strong electrostatic attraction and one antigen adsorbed by ligand exchange (BSA/alpha-casein); and one antigen adsorbed by weak electrostatic attraction and one antigen adsorbed by ligand exchange (myoglobin/alpha-casein). The images obtained by fluorescent microscopy of each system were similar to Fig. 9.

4. Discussion

The results indicate that antigen is distributed uniformily throughout vaccines through a cycle of de-aggregation and re-aggregation of the aluminum hydroxide adjuvant aggregates. The concentration of the antigen in the vaccines studied ranged from 4 to 30% of the adsorptive capacity of the aluminum hydroxide adjuvant. It is believed that the results of this study can be applied to other vaccines in which the

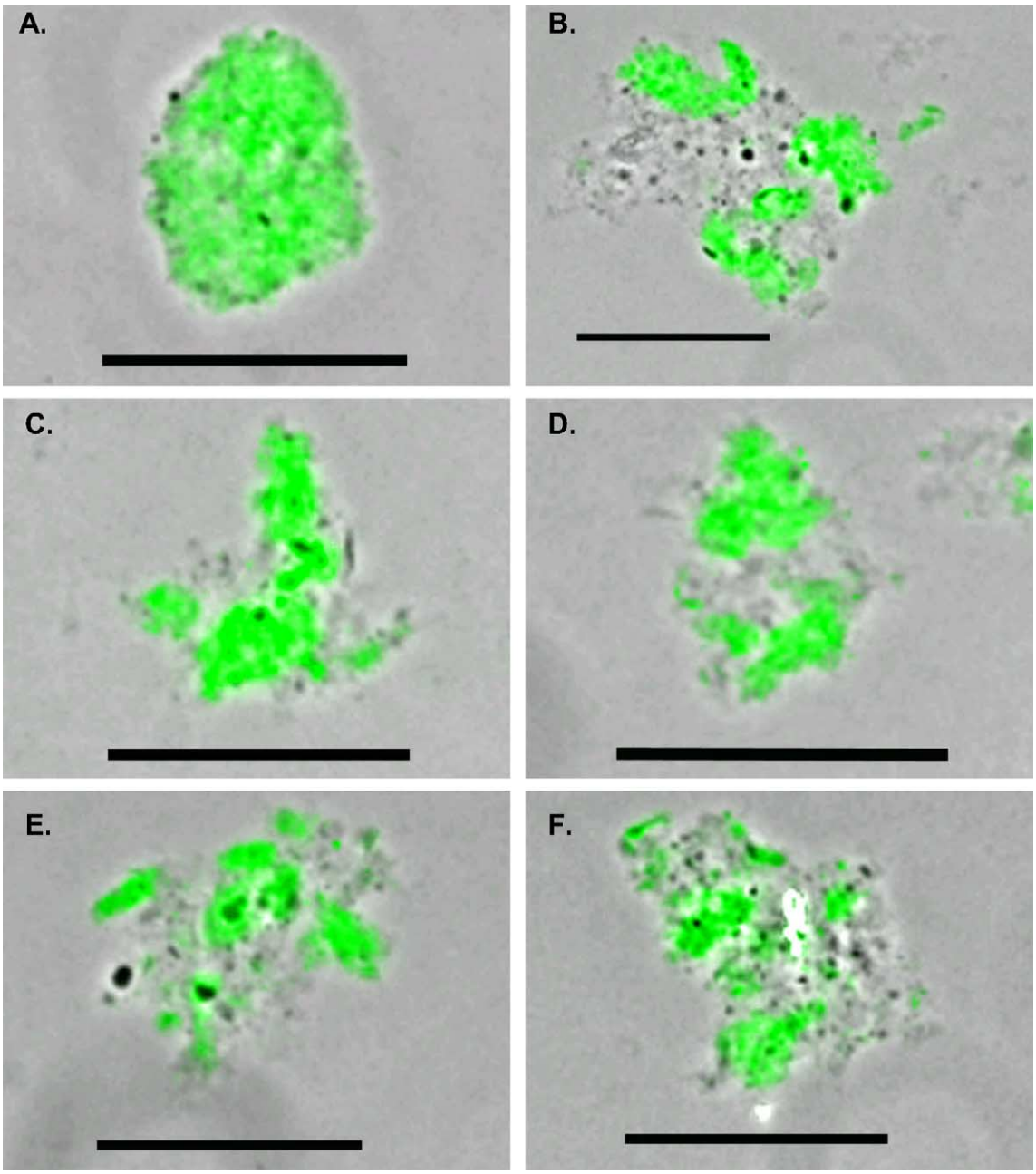

Fig. 8. Distribution of BODIPY FL-labeled alpha-casein in aluminum hydroxide when the antigen was adsorbed to 25% of the adjuvant and the mixture diluted with the remaining 75% of the aluminum hydroxide adjuvant: (A) initial adsorption of myoglobin to aluminum hydroxide adjuvant; (B) 1 min after addition of balance of aluminum hydroxide adjuvant to A with mixing; (C) 15 min of mixing; (D) 30 min of mixing; (E) 45 min of mixing; (F) 60 min of mixing. The bars represent 5 μm.

antigen concentration is less than 30% of the adsorptive capacity of the aluminum hydroxide adjuvant. This is likely to be the case in most vaccines as the dose of most antigens is in the microgram range. However, care should be taken before applying the results of this study to vaccines in which the antigen concentration exceeds 30% of the adsorptive capacity of the aluminum hydroxide adjuvant. This is most likely to occur in combination vaccines containing many antigens. When the total antigen concentration approaches or exceeds the adsorptive capacity of the aluminum hydroxide adjuvant it is likely that individual antigens will compete for adsorption. The antigens having a high affinity for the adjuvant will displace the antigens having a low affinity (8).

Aluminum hydroxide adjuvant from two sources was used in this study. Both aluminum hydroxide adjuvants were composed of very small fibrous primary particles that formed aggregates. When mixed, both aluminum hydroxide adjuvants produced uniform distribution of an adsorbed antigen

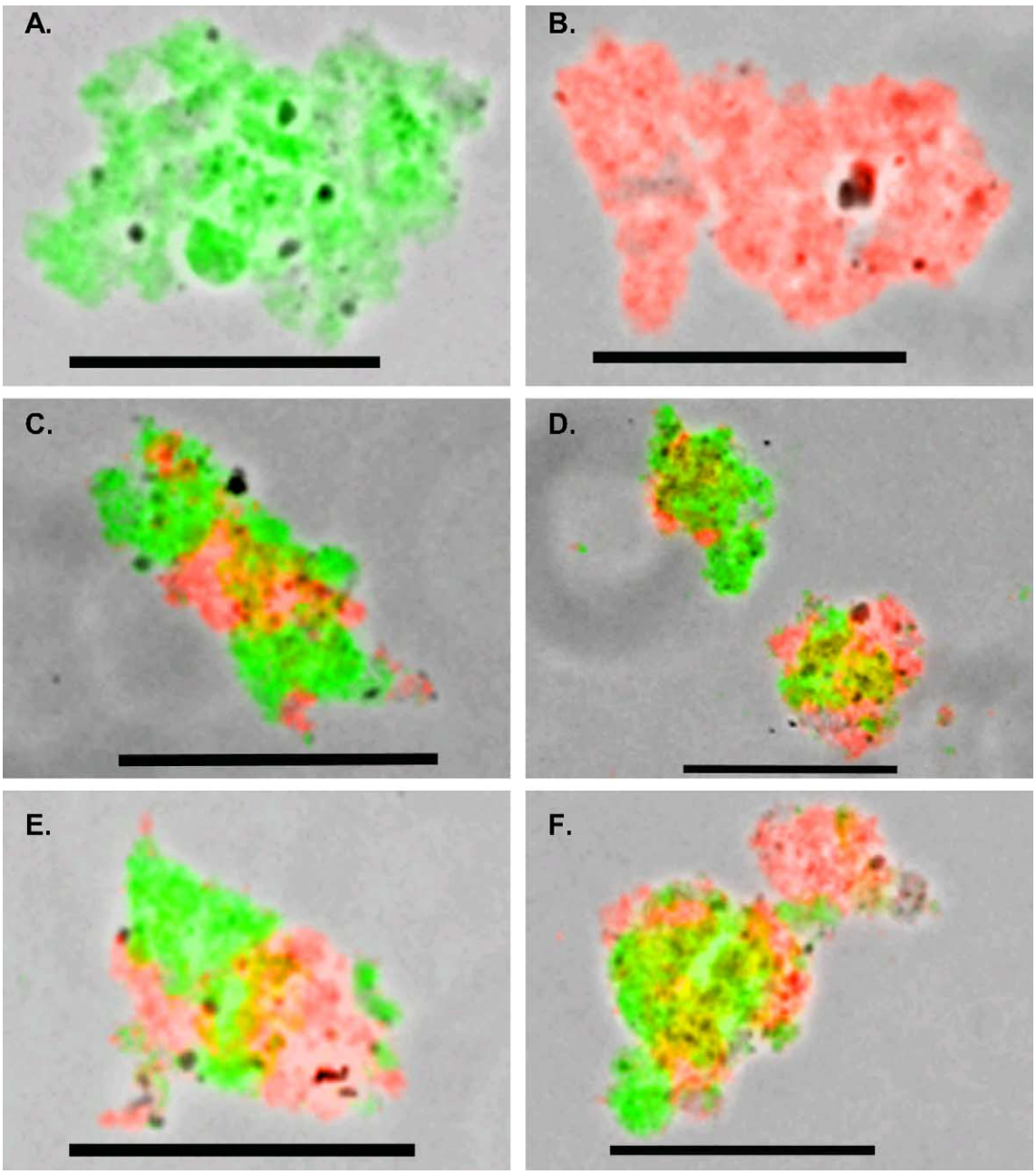

Fig. 9. Distribution of BODIPY FL-labeled bovine serum albumin adsorbed to aluminum hydroxide adjuvant and BODIPY TR-labeled bovine serum albumin adsorbed to aluminum hydroxide adjuvant in a combination vaccine: (A) BODIPY FL-labeled bovine serum albumin adsorbed to aluminum hydroxide adjuvant prior to combination; (B) BODIPY TR-labeled bovine serum albumin adsorbed to aluminum hydroxide adjuvant prior to combination; (C) 15 min after combination with mixing; (D) 30 min of mixing; (E) 45 min of mixing; (F) 60 min of mixing. The bars represent 5 μm.

by the same de-aggregation and re-aggregation mechanism. Thus, the morphology of aluminum hydroxide adjuvant is favorable for the uniform distribution of antigen as long as adequate mixing occurs.

The results of this study are consistent with an earlier study [15] that monitored the electrophoretic mobility when a negatively charged mono-valent vaccine was combined with a positively charged mono-valent vaccine. The combination vaccine initially exhibited a population having a negative electrophoretic mobility and a second population having a positive electrophoretic mobility. Upon mixing, the electrophoretic mobility of each population approached zero and the combination vaccine exhibited one population having an electrophoretic mobility of approximately zero. The de-aggregation and re-aggregation of adjuvant aggregates observed in the present study likely produced the change in electrophoretic mobility observed in the earlier study.

5. Conclusions

Aluminum hydroxide adjuvant exists in vaccines as aggregates of the fibrous primary particles. The adjuvant aggregates undergo a de-aggregation and re-aggregation process during mixing. This de-aggregation and re-aggregation process provides a mechanism that distributes antigens in mono-valent or combination vaccines throughout all of the adjuvant aggregates in the vaccine. Thus, content uniformity of the antigen in vaccines in which the antigen is adsorbed to aluminum hydroxide adjuvant should not be an issue as long as adequate mixing is provided during the production of the vaccine.

Antigens that are adsorbed by weak electrostatic attraction may elute when the vaccine is diluted as occurs upon the addition of additional aluminum hydroxide adjuvant. The eluted antigen may adsorb to the added adjuvant and thus facilitate uniform distribution of the antigen throughout the vaccine. The elution and re-adsorption mechanism operates in addition to the de-aggregation and re-aggregation mechanism.

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