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Elevated 7S Immunoglobulin and Acute Phase Proteins in Adjuvant-Injected Chickens[1] (39032)

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Associated with the potentiation of the immune response by Freund's complete adjuvant (FCA) are several factors which might account for adjuvanticity. At the injection site of adjuvant a granulomatous reaction occurs (1). Granuloma development is dependent on the incorporation of *Mycobacterium* and seems to be responsible for the prolonged synthesis of antibody (1). Furthermore, the development of delayed hypersensitivity (DHS) to antigens incorporated in the emulsion and the concomitant elaboration of products of activated thymus (T)-derived lymphocytes also may be involved in immune augmentation (2). In addition, we had noted greatly increased serum levels of 7S and other proteins in FCA-injected chickens. Therefore, we initiated a study on the characterization of these serum proteins and their relationships to accompanying granuloma formation and DHS.

Materials and methods. Adult white leghorn hens were given intramuscular injections in the thigh of 1 ml FCA which was prepared by emulsifying an equal volume of saline with Freund's incomplete adjuvant (FIA) (Difco) to which was added varying amounts of *Mycobacterium tuberculosis* (H37Ra, Difco). This adjuvant will be referred to as FIA-TB. In some experiments, FCA which contained 1 mg/ml of *Mycobacterium butyricum* (Difco) was supplemented with 10 mg/ml H37Ra. This adjuvant will be referred to as FCA-TB. Other chickens were injected with 1 ml of saline-FIA emulsions containing either 4×10^{10} killed *Bordetella pertussis* (Wyeth), or 0.8 mg killed *Nocardia rubra*, or 0.9 mg killed *Corynebacterium diphtheriae*, or 20 mg bovine serum albumin (BSA), or 0.4 mg *Mycobacterium tuberculosis* cell walls. These adjuvants will be referred to as FIA-PT, FIA-NR, FIA-BSA, and FIA-Walls, respectively. To induce acute phase proteins, chickens were given subcutaneous injections in the thigh of 6 ml of an equivolume mixture of turpentine–corn oil.

Granuloma development was estimated by measuring the circumference of the adjuvant-injected thigh. Delayed hypersensitivity was judged by intradermal injection of wattles with 0.1 ml either of a 1:10 dilution of old tuberculin (Parke-Davis), or 4.5×10^8 killed *B. pertussis*, or 20 μg *N. rubra* skin test antigen, or 20 μg killed *C. diphtheriae*. The increase in wattle thickness was measured with a caliper (Schnelltester, Germany).

Sera were electrophoresed on a Beckman Model R-101 Microzone electrophoresis cell for 20 min at 250 V in veronal buffer, pH 8.6, $\Gamma/2 = 0.075$. The cellulose acetate strips were stained with Ponceau S, cleared, and tracings were made with a densitometer (Quick Scan, Helena Laboratories) equipped with an integrator for determination of the area under each peak.

The levels of the major chicken immunoglobulin (7S Ig) were determined by radial immunodiffusion (3) using rabbit anti-chicken 7S Ig (4). The chicken 7S Ig used for preparing a standard curve was obtained as previously described (5).

Ultracentrifugation of serum diluted 1:1 in borate buffer (pH 8.2) was performed with a Spinco Model E analytical ultracentrifuge equipped with schlieren optics, phase plate, and a temperature control unit set at 23°. Centrifugation was at 59,720 rpm.

Results. Granuloma formation and DHS. Groups of four chickens each were injected with either FCA-TB, FIA-TB, FIA-PT, or FIA-NR. All birds developed large granulomas, which became visible by 7–14 days and reached maximum size 28–35 days fol-

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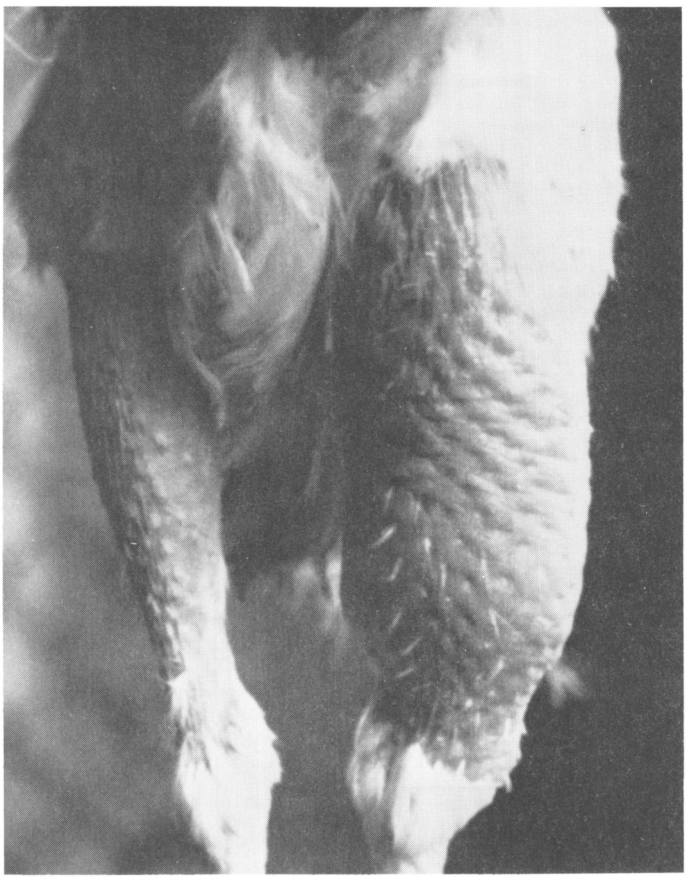

FIG. 1. A granuloma in the chicken produced 29 days after injection with FCA-TB.

lowing injection. By 60 days, most of the granulomas had regressed to small, hard nodules. In Fig. 1 is shown a representative granuloma 29 days after the bird had been injected with FCA-TB. The thigh bearing this granuloma increased in circumference from 9.5 to 13 cm. This represented a 37% increase in circumference or approximately a 280% increase in volume (calculated assuming the granuloma was a sphere). When other groups of four birds were injected with either FIA-CD, FIA-Walls, FIA-BSA or with saline suspensions of either *B. pertussis* or *M. tuberculosis*, granulomas were not observed (Table I).

Detectable cutaneous DHS occurred only in those animals in which granulomas had developed (Table I). These reactions were maximum 3–4 days after injection of antigen, and greater than ten-fold increases in wattle thicknesses were observed in some animals. In such intense reactions, edema occurred in the uninjected contralateral wattle, and the increases in both wattles were summed (Table I). The uninjected wattle averaged 0.8 mm in thickness. The DHS reactions were distinct from the Arthus reaction in the chicken wattle, which has been observed to peak at 2–4 hr (6).

Electrophoretic analyses. Sera from the birds injected above were analyzed by electrophoresis. Similar to the development of DHS, abnormal protein patterns were detected only in those birds in which granulomas had appeared. The electrophoretic patterns of four representative birds in Fig. 2 clearly show that 10 days after injection with FCA-TB the concentrations of the albumin were markedly decreased and the concentrations of α-, β-, and γ-proteins

TABLE I. RELATIONSHIPS BETWEEN GRANULOMA SIZE, DELAYED HYPERSENSITIVITY, AND SERUM PROTEINS IN CHICKENS 29 DAYS AFTER INJECTION OF VARIOUS ADJUVANTS.

Adjuvant	Bird	Increase in granuloma circumference (%)	Delayed hypersensitivity Increase in wattle thickness (mm) on day following skin test (Day) 1	2	3	4	5	6	Serum electrophoretic pattern
FCA-TB[a]	1876	37	2.7	5.5	8.4	6.3	3.1	1.4	Abnormal[b]
	1877	65	2.9	10.4	12.0	14.1	7.5	3.8	Abnormal
	1878	53	3.0	10.6	15.1	14.8	14.2	11.5	Abnormal
	1879	30	2.2	3.3	4.2	6.8	1.9	1.3	Abnormal
FIA-PT	1880	17	2.1	4.8	6.3	3.7	1.6	1.2	Abnormal
	1881	19	0.9	2.0	2.5	2.2	1.3	0.9	Abnormal
	1882	44	1.2	4.1	3.1	1.2	0.9	0.9	Abnormal
	1883	10	1.2	3.7	2.6	1.2	1.1	0.8	Abnormal
FIA-NR	1896	17	2.4	6.8	6.2	5.0	1.8	0.8	Abnormal
	1897	29	2.0	3.5	5.8	4.3	2.3	2.2	Abnormal
	1898	25	1.2	4.0	4.3	2.2	1.1	0.9	Abnormal
FIA-CD[c]	1892	0	0	0	0.1	0.2	0.1	0.2	Normal
	1893	0	0	0	0	0	0	0	Normal
	1894	0	0	0	0	0.1	0	0	Normal
	1895	5[d]	0.6	0.2	0.2	0.1	0.1	0	Normal

[a] FCA = Freund's complete adjuvant; FIA = Freund's incomplete adjuvant; TB = *Mycobacterium tuberculosis*; PT = *Bordetella pertussis*; NR = *Nocardia rubra*; CD = *Corynebacterium diphtheriae*.

[b] Increased α-, β-, and γ-proteins; decreased albumin concentration.

[c] Similar results were obtained for birds injected with FIA-walls and *M. tuberculosis* or *B. pertussis* in saline.

[d] Within limits of error due to measurement.

were increased. At this time, the fast-moving γ-protein appeared to have restricted heterogeneity. At 25 days the concentrations of the γ-proteins were greatly increased, and they appeared to be electrophoretically-heterogenous. The albumin concentration was still depressed, and the α- and β-proteins were still elevated at 25 days. The "homogeneous" γ-protein was masked by the increased heterogeneous γ-globulins at 25 days, since it was still present after removal of 7S Ig by precipitation with 18% Na_2SO_4. At 0 day, the distinct bands seen at the origin are lipid artifacts seen in the sera of unfasted birds. The temporal synthesis of these proteins is shown by the electrophoretic patterns of sera obtained at 5-day intervals from a single bird (Fig. 2). These protein changes were maximum at about 20–35 days. By 50 days the patterns appeared to have returned to normal except for an elevated concentration of the β-protein.

Examination of the electrophoretic patterns indicated that the degree of serum alterations appeared to be related to the quantity of TB in FIA. Groups of four birds each were injected with FIA supplemented with *M. tuberculosis* ranging from 0 to 15 mg/ml emulsion. Typical serum patterns of a single representative bird in each group, obtained 28 days after injection, are shown in Fig. 3. As the dose of TB increased, the protein changes became more evident. Chickens injected with FIA had normal serum protein patterns. In addition, four birds injected with 20 mg of BSA in FIA, a highly immunogenic dose, failed to show these changes over a 64-day period.

Ultracentrifugation. The sedimentation patterns of a normal chicken serum and a serum from a bird which had received FCA-TB are shown in Fig. 4. The serum from the adjuvant-injected bird had a new peak having a sedimentation coefficient of about 11–12S and a greatly increased 7S peak. The observed decrease in the sedimentation rate of the 7S proteins, from 6.4S to 5.6S (Fig. 4), probably was due to the Johnston–Ogsten effect and to the fact that the sedimentation coefficient is inversely

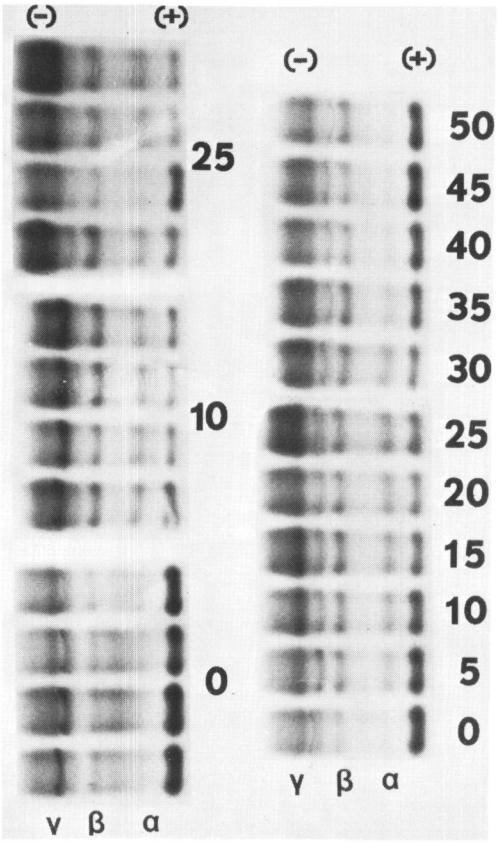

FIG. 2. Left. Electrophoretic patterns of sera obtained from four chickens (Nos. 1876, 1877, 1878, and 1879 from bottom to top in each panels) on the indicated days following injection of FCA-TB. Right. The electrophoretic patterns of serum at 5-day intervals from chicken No. 1876.

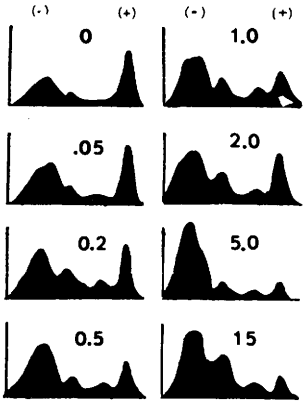

FIG. 3. Representative electrophoretic changes in serum proteins induced in chickens injected with FIA containing various concentrations (0–15 mg) of *Mycobacterium tuberculosis*.

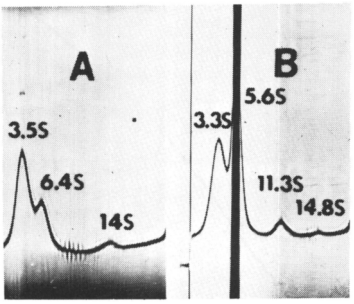

FIG. 4. Ultracentrifugal pattern of a normal serum (A) and a serum obtained 32 days from a chicken after injection with FCA-TB (B). Pictures were taken 32 min after reaching speed (59,720 rpm). Sedimentation is from left to right.

proportional to protein concentration. When these sera were diluted, the sedimentation rates approached 7S. The increased concentration of the 7S proteins was estimated by the method of half-heights. In one observation, the average increase in 4 birds injected with FIA alone compared to four birds injected with FIA containing 0.5 mg TB was 100%. A 20% increase in the 3–4S proteins also was observed, despite the decrease in albumin observed by electrophoresis. This was due to the presence of approximately 4–5S α- and γ-mobility acute phase proteins, which will be the subject of a subsequent report.

Radial immunodiffusion. Shown in Table II are the levels of 7S Ig at 5-day intervals in representative birds injected with FCA-TB, FIA-PT, and FIA-NR. The concentrations were maximum 20–35 days after injection, and some sera increased nearly 200%. The 7S Ig levels decreased gradually after 35 days, however, they did not return to normal by 50 days. Birds injected with 20 mg BSA in FIA failed to show elevated 7S Ig levels over a 64-day period.

Adsorption. The 20-day serum from bird 1880 (Table II), which had been injected with FIA-PT, was diluted 1:4 in borate buffer and was adsorbed with 4.6×10^{11} lyophilized *B. pertussis* cells for 1 hr at 37° followed by overnight refrigeration. The bacteria were pelleted by centrifugation and two additional adsorptions were performed. The agglutination titer dropped from 1:1280 to 1:10 after the adsorptions, but the concentration of 7S Ig decreased only from

TABLE II. INCREASE IN 7S Ig IN REPRESENTATIVE ADJUVANT-INJECTED BIRDS AS DETERMINED BY RADIAL IMMUNODIFFUSION.

Days after injection	Adjuvant: Freund's complete + *M.* tuberculosis		Freund's incomplete + *B.* pertussis		Freund's incomplete + *N.* rubra	
	1876[a]	1878	1880	1883	1896	1897
0	12.9 (0)[b]	12.3 (0)	14.4 (0)	9.4 (0)	14.1 (0)	13.0 (0)
5	12.0 (−7)	15.9 (29)	14.0 (−3)	10.2 (8)	19.5 (38)	13.0 (0)
10	14.7 (14)	17.7 (44)	22.4 (55)	19.9 (111)	24.3 (72)	15.9 (22)
15	17.8 (40)	23.1 (88)	29.6 (105)	19.4 (106)	36.9 (162)	22.5 (73)
20	21.1 (64)	30.9 (151)	32.6 (126)	23.1 (145)	39.0 (177)	21.9 (68)
25	30.6 (137)	29.7 (141)	31.0 (115)	22.1 (135)	41.7 (195)	21.8 (68)
30	30.0 (133)	30.0 (144)	28.3 (96)	17.7 (88)	39.3 (179)	20.9 (61)
35	33.3 (158)	25.5 (107)	30.0 (108)	18.2 (93)	36.0 (155)	27.6 (112)
40	27.9 (116)	26.7 (117)	26.1 (81)	15.8 (68)	29.7 (111)	29.4 (126)
45	23.1 (79)	23.4 (90)	(−)[c] (−)	15.0 (59)	25.4 (80)	28.6 (120)
50	19.5 (51)	(−) (−)	24.9 (73)	12.4 (32)	25.6 (82)	24.6 (89)

[a] Bird number.

[b] Milligrams of 7S Ig per millimeter of serum. Number in parenthesis represents % increase over normal.

[c] Not tested.

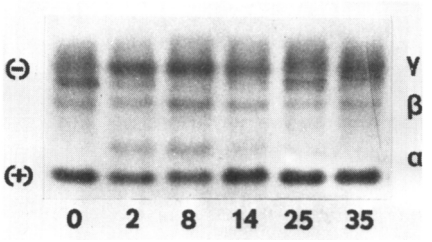

FIG. 5. Changes in the electrophoretic patterns of sera obtained on the indicated days from a chicken which had been injected with turpentine-oil.

32.6 to 29.8 mg/ml as determined by radial immunodiffusion. The 35-day serum from bird 1876 (Table II and Fig. 2) which had been injected with FCA-TB was diluted 1:4 and adsorbed three times with 25 mg of desiccated *M. tuberculosis* (H37Ra, Difco) in a similar manner. The 7S Ig level decreased from 33.3 to 32.7 mg/ml. Also, the 7S Ig level was the same before and after adsorption as determined in the ultracentrifuge and by electrophoresis.

Acute phase (AP) proteins. As a result of injury or inflammation, the concentrations of numerous serum proteins increase in man and other animals (7). Known collectively as acute phase proteins, probably the most notable of these is C-reactive protein. Four birds were injected with a mixture of turpentine-oil, a procedure known to induce AP proteins in the rat (8). Similar to the changes observed in chickens bearing intense inflammatory responses (the adjuvant-induced granulomas), the concentration of albumin decreased and α-, β-, and γ-mobility proteins increased (Fig. 5). However, unlike the adjuvant-induced changes, these patterns were maximum approximately 1 wk after injection and diminished rapidly thereafter. No increase in heterogeneous, slow-migrating proteins was observed up to 35 days, even when elevated levels of α-, β-, and γ-mobility proteins were maintained by repeated, weekly injections of turpentine-oil. Also, in the ultracentrifuge no increase in the 7S proteins was observed.

Discussion. In contrast to the foreign-body granuloma, the allergic granuloma is thought to involve a local DHS reaction based on the following recent observations: The allergic granuloma shows specificity (9) and anamnesis (10); it can be passively transferred with cells (11); and it contains cells capable of releasing macrophage migration inhibition factor (12). The large granulomas observed were probably of the allergic type, since DHS could be elicited only in birds which developed granulomas. Furthermore, the adjuvant-induced granulomas are smaller in thymectomized birds (13).

It is noteworthy that elevated 7S Ig levels and acute phase proteins appeared only when adjuvants which induced DHS and allergic granuloma formation were used. Thus, the mechanism of increased 7S Ig synthesis seems to be related to adjuvant-induced allergic granuloma formation. Evidence exists for antibody synthesis within adjuvant-induced granulomas. White (13) reported the formation of large granulomas 3 weeks after subcutaneous injection of human serum albumin (HSA) in FCA. As measured by the Farr method, there was a biphasic antibody response. The first phase of antibody synthesis was probably splenic, and it peaked at 8–12 days. This was followed by a slow, progressively increasing second phase of anti-HSA antibody synthesis which occurred within the granuloma.

The following model is suggested to account for the observation of elevated 7S Ig and AP proteins following injection of FCA in the chicken. Initially, the deposition of the water-in-oil emulsion initiates the formation of a small, undetectable foreign body granuloma. The presence of *M. tuberculosis*, however, initiates a local DHS response. Circulating "sensitized" T lymphocytes migrate into the region and, in contact with antigen, release soluble factors. These products might account, in part, for the attraction and proliferation of lymphocytes and macrophages, resulting in enlargement of the granuloma. Local bursa-derived (B) lymphocytes are activated, perhaps by products of the activated T lymphocytes and intense 7S Ig synthesis occurs, which results in the greatly elevated "nonspecific" serum 7S Ig levels. The inflammatory response is accompanied by tissue damage and necrosis, which leads to the appearance of AP proteins in the blood.

The failure to adsorb the 7S Ig indicated that the Ig was not directed to surface antigens of the bacteria. Humphrey (14) noted similar, but less dramatic elevated serum γ-globulin levels in rabbits injected with FCA. In more exhaustive adsorption experiments, he showed that the increased Ig was not directed to internal mycobacterial antigens as well and suggested, as we believe here, that the Ig was "nonspecific." Work is in progress in search of a T cell product(s) capable of promoting antibody synthesis in a nonspecific manner similar to that which has been described previously (15, 16). The purification and characterization of the AP proteins will be the subject of another paper.

Summary. Injection of chickens with FCA or FIA supplemented with either *Bordetella pertussis* or *Nocardia rubra* induced greatly increased serum levels of 7S Ig and proteins with α-, β-, and γ-mobility in electrophoresis. The serum protein changes were correlated with the development of DHS and the formation of a large allergic granuloma. The 7S Ig was considered "nonspecific" since it was not adsorbed with the bacterial cells. The α-, β-, and γ-mobility proteins were identified as acute phase proteins as they also were induced by injection of chickens with turpentine–oil; however, increased serum levels of 7S Ig were not similarly increased.

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