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The Influence of Adjuvants on the Immunological Response of the Chicken

II. EFFECTS OF FREUND'S COMPLETE ADJUVANT ON LATER ANTIBODY PRODUCTION AFTER A SINGLE INJECTION OF IMMUNOGEN

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Summary. A study has been made in the chicken of the late phase (after the 21st day) of the immunological response to a single intramuscular injection of a soluble protein antigen, human serum albumin (HSA), incorporated in a water-in-oil emulsion with or without heat-killed *Mycobacterium tuberculosis*.

Birds injected with a water-in-oil emulsion showed, after a fall from the initial primary response peak (8–12th day), a second rise in antibody production beginning at the 21st day. Control birds injected with HSA in 0·15 M NaCl failed to show this second rise. The peak of the second phase of antibody production in these experiments fell between the 42nd and 59th days. The results of one experiment showed that in birds injected with Freund's complete adjuvant the average second peak level was 100-fold higher than the average first peak level. However, the second peak level of antibody reached was much higher in birds injected with HSA in Freund's complete adjuvant than in those injected with a simple water-in-oil emulsion.

The avidity of anti-HSA present in the second phase of antibody production by birds injected with HSA in water-in-oil emulsions has been compared with the avidity of anti-HSA produced during the first phase (10th day). The avidity of sera obtained during the second phase of anti-HSA production by birds given water-in-oil emulsions with or without added *M. tuberculosis* was much greater than that of sera obtained during the first phase.

The anatomical site of antibody production during the second phase of antibody production has been investigated. The findings suggest that, whereas antibody production during the first phase is in the spleen and probably other lymphoid tissues, much of the antibody produced during the second phase may be from the granuloma produced at the site of injection of the water-in-oil emulsion.

INTRODUCTION

The mode of action on the humoral immune response of Freund's complete adjuvant (protein antigen in water-in-oil emulsion with added mycobacteria) can be regarded as having two components: the slow release of the immunogen from the watery droplets of the emulsion and the specific adjuvant effect of mycobacteria or their contained peptidoglycolipids (Fischel, Kabat, Stoerk and Bezer, 1952; White, Bernstock, Johns and Lederer,

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1958; White, Jollès, Samour and Lederer, 1964). More recently it has been shown (Steinberg, Munro, Fleming, French, Stark and White, 1970) that a wide variety of commonly-used adjuvants, including Freund's complete and incomplete mixture, fail to increase the level of antibody at the peak of the early primary response (days 8–12). This communication deals with the influence of Freund's complete and incomplete adjuvant on antibody production by the chicken during the later phases of the response to a single injection of human serum albumin (HSA). Whereas the antibody response of birds which receive an intravenous injection of HSA has largely terminated by the 18th day, in birds which receive HSA in water-in-oil emulsion or Freund's complete mixture the initial fall of antibody level from the peak (8–12 days) is succeeded by a slow and prolonged rise to a second peak. The time course of the later phase of this biphasic response, the changes in avidity of the antibody produced, the antibody content of various lymphoid tissues and the accompanying immuno-histological findings are also described.

MATERIALS AND METHODS

Animals

Commercial White Leghorn hybrid birds (Thornber 606 and 808) aged 10–16 weeks were used.

Antigen and adjuvants

Human albumin (HSA) in doses of 40 μg was given in 0·15 M NaCl, or in water-in-oil emulsions with or without heat-killed *Mycobacterium tuberculosis* (5 mg/dose). The mixtures were prepared as described previously (Steinberg *et al.*, 1970).

Measurement of circulating antibody levels

The methods for obtaining serum samples and estimating the antigen binding capacity (ABC_{30} in μg/ml) by the Farr technique were as previously described (Steinberg *et al.*, 1970). The samples were stored at −20° for up to 8 weeks before antibody levels were estimated.

Estimation of avidity

Sera obtained from birds 10, 35 and 49 days after the injection of antigen (40 μg HSA) in water-in-oil emulsion, in Freund's complete adjuvant, or in 0·15 M saline were examined for the avidity of their reaction with HSA. The antigen binding capacity of each serum was estimated by the Farr test, the sera being compared on the basis of their ability to bind 20 per cent of the antigen present (ABC_{20}). The ABC_{20} for each serum was estimated with four separate concentrations of [^{131}I]HSA, namely 1·5, 0·6, 0·3 and 0·03 μg/ml or 1·5, 0·6, 0·24 and 0·024 μg/ml. The dilutions of serum were made in a 1 : 10 dilution of normal chicken serum in 0·15 M saline. After an initial fourfold dilution (0·2 ml test serum in 0·6 ml diluent) further dilutions with thorough mixing were made by transfer of 0·5-ml volumes into 1·5 ml diluent in $5 \times \frac{3}{8}$ in. test tubes. Dilutions were made to $1 : 2^{14}$. Four dilutions of each serum appropriate for each antigen concentration were set up for the Farr test. The reacting volumes and times of reaction were as described previously (Steinberg *et al.*, 1970). The final antigen concentrations in the reaction mixtures were 1, 0·4, 0·2 and 0·02 μg/ml or 1·0, 0·4, 0·16 and 0·016 μg/ml.

For each serum, the antigen concentration in the reaction mixture was plotted

against the ABC_{20} on semilog paper with the antigen concentration on the logarithmic axis. The concentration of antigen could then be determined at which the ABC_{20} equalled half the ABC_{20} at the highest antigen concentration (1 μg/ml). The values obtained were taken as a measure of the antibody avidity.

Examination of tissues by immunofluorescence for the cellular production of antibody

An injection of 40 μg HSA in 0·15 M NaCl, Freund's complete or incomplete adjuvant was made into the right pectoral muscles of 10–16-week-old chickens. At varying time intervals after antigen injection the birds were killed with intravenous sodium pentobarbitone. Pieces of spleen, liver, bursa, thymus, lung, skin, bone marrow, caecal tonsil and granulomatous tissue (when present macroscopically) from the site of intramuscular injection, were removed and snap frozen at $-70°$. The tissues were sectioned at 4 μ thickness in a cryostat at $-20°$, mounted on microscope slides and, after thawing to room temperature, fixed in absolute methanol for 15 minutes. The sections were then stained to demonstrate the presence of anti-HSA by the 'sandwich' modification of the fluorescent antibody staining technique (Coons, Leduc and Connolly, 1955). After dipping the sections in phosphate buffered saline (PBS) a drop of HSA in 0·15 M NaCl (2 mg/ml) was applied to each section. After 30 minutes the sections were washed for 5 minutes in PBS and a drop of fluorescein isothiocyanate-labelled rabbit anti-HSA serum then applied to each section. After a further 30 minutes the sections were washed in PBS for 10 minutes and then mounted in PBS. Control sections were stained with fluorescein labelled rabbit anti-HSA only. Sections treated by this method stained HSA present in the section. Cells showing fluorescence only in the sections stained by the 'sandwich' technique and not in the control sections were considered to be anti-HSA containing cells. They were examined using a fluorescence microscope fitted with a mercury vapour light source (Osram HBO 200).

Measurement of anti-HSA content of tissues

Three chickens were injected with 40 μg HSA in Freund's complete adjuvant into the right pectoral muscle. They were killed 54 days later with intravenous sodium pentobarbitone. Small pieces of tissues to be investigated were removed and weighed. The tissues were then immediately frozen to $-70°$ and stored at this temperature for two months. Anti-HSA was extracted from the tissue cells initially by thawing the tissue rapidly at 37° and then rapidly refreezing to $-70°$ on two occasions. On rewarming the tissues to room temperature 1·0 ml 0·15 M NaCl was added to each block of tissue and the probe of an ultrasonicator (Soniprobe Type 1130 Dawe Instruments, Ltd, Concord Road, London, W.3) introduced into the fluid at a frequency of 20 kHz and a power output of approximately 50 W for 30 seconds. The resulting suspension was centrifuged at 1000 ***g*** for 10 minutes and the supernatant collected for measurement of its antigen (HSA) binding capacity. The value obtained for each tissue extract was expressed as the amount of HSA binding capacity extractable from 1 g of tissue.

RESULTS

Tables 1, 2 and 3 include the results of three groups of experiments which were planned to explore the conditions under which Freund's complete adjuvant influences the antibody response to HSA in the chicken.

COMPARISON OF THE EFFECT OF HSA IN FREUND'S COMPLETE ADJUVANT WITH HSA IN 0·15 M NaCl ON SERUM ANTIBODY LEVELS

As seen from Table 1 control birds injected with 40 μg (HSA) in 0·15 M NaCl produced little or no circulating anti-HSA (ABC_{30} below 0·002 μg/ml) during the period 4–21 days after intramuscular injection. Birds receiving the same dose of HSA in Freund's complete adjuvant (including 5 mg heat-killed *M. tuberculosis*) all produced measurable levels of anti-HSA within the period 4–14 days. The time at which the peak value was seen varied in different birds (from day 7 to day 11). On the 14th day the values had fallen in all birds receiving adjuvant below that of the peak but comparatively high levels of anti-HSA were again found at 21 days. As compared with the controls the first peak of the birds receiving Freund's complete adjuvant showed an increase, at least four-fold over the average value for the control birds and the second peak at least a 250-fold increase over control birds. For the birds receiving Freund's complete adjuvant the ratio of the average anti-HSA levels of the second peak to that of the first peak was 34.

TABLE 1

TIME COURSE OF ANTIBODY RESPONSE OF MALE 13-WEEK-OLD CHICKENS TO A SINGLE INJECTION OF 40 μg HSA IN FREUND'S COMPLETE ADJUVANT COMPARED WITH CONTROLS

Experiment treatment	Animal No.	Antibody level ABC_{30}(μg/ml)					
		4 days	7 days	9 days	11 days	14 days	21 days
40 μg HSA in w/o emulsion with 5 mg heat-killed *M. tuberculosis*	41	<0·002	<0·002	<0·002	0·045	0·040	2·4
	40	<0·002	0·051	<0·002	<0·002	<0·002	5·1
	39	<0·002	0·375	0·14	0·012	0·009	6·9
	37	<0·002	<0·002	0·12	<0·002	<0·002	7·5
(Four controls) 40 μg HSA in 0·15 M NaCl	47, 48, 49, 50	<0·002	<0·002	<0·002	<0·002	<0·002	<0·002

COMPARISON OF TWO ANTIGEN DOSE LEVELS (40 μg AND 40 mg) OF SERUM ANTIBODY PRODUCTION UP TO 21 DAYS AFTER INJECTION OF HSA IN FREUND'S COMPLETE OR INCOMPLETE EMULSION OR IN SALINE SOLUTION

As seen from Table 2 all groups of birds attained a peak value for anti-HSA during the primary response period. The peak was at either the 10th or 12th day in both the control sets of birds. There was no significant difference in the time or in the average antibody value of the peak between the groups of birds receiving 40 μg HSA in water-in-oil emulsions (with or without the addition of 5 mg *M. tuberculosis*) and the group receiving 40 μg HSA in 0·15 M NaCl.

Table 2 also shows that when the amount of HSA in the injection mixture was increased from 40 μg to 40 mg the average peak anti-HSA level of the first response was increased sixty-fold in those birds receiving HSA in 0·15 M NaCl. When the same increase of antigen was used for injections in Freund's complete adjuvant a twenty-eight-fold increase was observed. Thus increase in dose of antigen increases the antibody level at the peak but Freund's complete adjuvant has no appreciable effect in increasing peak antibody levels at either 40 μg or 40 mg dose level of antigen.

In control birds receiving HSA in 0·15 M NaCl at both levels of antigen dosage (40 μg and 40 mg HSA) the level of antibody fails to rise for a second peak of antibody after 21

TABLE 2

TIME COURSE OF SERUM ANTIBODY LEVELS OF FEMALE 10-WEEK-OLD CHICKENS AFTER A SINGLE INJECTION OF 40 μg OR 40 MG HSA IN FREUND'S COMPLETE ADJUVANT COMPARED WITH CONTROLS

Experimental treatment	Animal No.	Serum antibody level: ABC_{30} (μg/ml) at various time intervals after injection					
		4 days	7 days	10 days	12 days	18 days	21 days
40 μg HSA in w/o emulsion with 5 mg heat-killed *M. tuberculosis*	58	<0·006	0·2	2·85	2·6	0·16	7·5
	61	<0·006	0·83	1·65	1·25	0·42	1·94
	62	<0·006	<0·006	2·85	3·2	0·75	3·15
40 μg HSA in w/o emulsion	54	<0·006	0·51	3·9	3·3	3·45	2·7
	59	<0·006	<0·006	2·25	0·84	0·18	0·42
	63	<0·006	0·3	3·15	4·35	0·72	3·0
40 μg HSA in 0·15 M NaCl	57	<0·006	1·25	2·6	7·35	0·26	0·27
	52	0·23	<0·006	0·92	0·87	0·3	<0·006
	55	<0·006	<0·006	3·75	2·85	0·64	0·27
40 mg HSA in w/o emulsion with 5 mg heat-killed *M. tuberculosis*	60	<0·006	12·6	3·75	2·25	0·88	3·15
	64	<0·006	16·8	12·0	12·6	10·2	13·8
	65	<0·006	0·19	2·45	1·8	0·18	1·5
40 mg HSA in 0·15 M NaCl	51	<0·006	0·68	3·9	2·7	0·45	<0·006
	53	<0·006	13·2	10·8	9·6	2·4	1·38
	56	<0·006	63·6	>75·0	73·5	4·5	0·9

days. However, the level of antibody in birds receiving antigen at either dosage (40 μg and 40 mg) in Freund's complete adjuvant rose abruptly after the 18th day. In those birds receiving HSA (40 μg) in Freund's incomplete adjuvant the serum level also rose after the 18th day, although the observed rise was less than that of the birds receiving antigen in Freund's complete adjuvant.

THE LATE PHASE OF THE ANTIBODY RESPONSE FOLLOWED FOR 59 DAYS AFTER INJECTION OF HSA IN FREUND'S COMPLETE ADJUVANT OR IN WATER-IN-OIL EMULSION OR IN SALINE

The experiments shown in Table 3 comprised two sets of birds, 10 and 16 weeks of age at the start of the experiment. In the 10-week-old birds the use of Freund's complete adjuvant led to an average peak value (ABC_{30}) of anti-HSA of 23·25 μg/ml (peak at the 12th day). In the control birds which received antigen in 0·15 M NaCl the average peak value was 2·04 μg/ml (peak at the 12th day).

The fall from the first peak of the primary response was slow in those birds receiving antigen in Freund's complete adjuvant. The lowest level was reached between the 22nd day and the 33rd day after injection. From the 40th day onwards in the sole surviving bird the serum antibody level rose strikingly to 45 μg/ml at the 43rd day. From the 22nd day onwards the serum antibody level of the control birds (receiving HSA in 0·15 M NaCl) fell to immeasurably low levels.

In the group of 16-week old birds observations of serum antibody levels were continued up to the 59th day after injection. As seen from Table 3 the average serum antibody values (ABC_{30}) of the first peak of the response in birds receiving 40 μg HSA in Freund's complete adjuvant was 5·49 μg/ml, in the birds receiving HSA in water-in-oil emulsion was 9·61 μg/ml and in birds receiving HSA in 0·15 M NaCl was 3·06 μg/ml. The birds receiving Freund's complete adjuvant showed a continuing rise of serum antibody level to a peak at 42–49 days. The average of the peak (ABC_{30}) values was 413 μg/ml (in one bird the serum antibody values continued to rise up to the 59th day).

TABLE 3

TIME COURSE OF SERUM ANTIBODY LEVELS OF FEMALE 10- AND 16-WEEK-OLD CHICKENS AFTER A SINGLE INJECTION OF 40 μg HSA IN FREUND'S COMPLETE ADJUVANT COMPARED WITH CONTROLS

Experimental treatment	Age (weeks)		Day 12	15	22	26	28	33	35	40	43
40 μg HSA in w/o emulsion with 5 mg heat-killed *M. tuberculosis*	10	Mean ABC_{30} (μg/ml)	23·25	9·68	8·46	5·76	7·07	5·88	5·7	5·4	45·0
	10	No. of birds	4	4	4	3	3	2	2	1	1
40 μg HSA in 0·15 M NaCl		Mean ABC_{30} (μg/ml)	2·04	1·18	0·48	<0·006	<0·006	<0·006	<0·006	<0·006	<0·006
		No. of birds	4	4	4	3	3	2	2	1	1

			Day 10	18	21	35	40	42	49	59
40 μg HSA in w/o emulsion with 5 mg heat-killed *M. tuberculosis*	16	Mean ABC_{30} (μg/ml)	5·49	5·89	5·28	141·7	180·8	412·8	413·4	323·0
		No. of birds	5	5	5	5	5	5	5	4
40 μg HSA in w/o emulsion	16	Mean ABC_{30} (μg/ml)	9·61	6·02	3·54	21·02	15·98	27·7	19·0	19·1
		No. of birds	5	5	5	5	5	5	5	5
40 μg HSA in 0·15 M NaCl	16	Mean ABC_{30} (μg/ml)	3·06	0·83	1·48	0·39	<0·06	1·19	<0·06	0·73
		No. of birds	5	5	5	5	5	5	5	3

The same delayed rise of antibody levels to a second peak was observed in the group of birds receiving antigen in water-in-oil emulsion, but under these circumstances the peak was slightly earlier (42nd day). The levels of antibody at the peak averaged 27·7 μg/ml as compared to 413·4 μg/ml in the birds receiving HSA in Freund's complete adjuvant. No comparable rise in serum antibody levels occurred in birds receiving HSA in 0·15 M NaCl.

INFLUENCE OF FREUND'S COMPLETE ADJUVANT ON THE AVIDITY OF ANTI-HSA PRODUCED DURING THE SECOND PHASE OF THE ANTIBODY RESPONSE

The avidity of anti-HSA in serum samples taken from five birds on the 10th, 35th and 49th days after injection of 40 μg HSA in either 0·15 M NaCl or water-in-oil emulsion with or without *M. tuberculosis* was measured by observing the variation of antigen binding capacity with decreasing antigen concentration. Antigen binding capacity, ABC_{20}, was measured by the method of ammonium sulphate precipitation of antigen–antibody complexes (Farr, 1958). The ABC_{20} of the sera measured at varying [^{131}I]HSA concentrations (0·016–1·0 μg/ml) are given in Table 4. Table 4 also provides information on the antigen

TABLE 4

EFFECT OF DECREASING ANTIGEN CONCENTRATION ON THE ABC_{20} OF SERA FROM BIRDS GIVEN ANTIGEN WITH AND WITHOUT ADJUVANT

Stimulus	Day after injection	Antigen concentration (μg/ml) where ABC_{20} = 50 per cent of ABC_{20} at 1·0 μg/ml
40 μg HSA in Freund's complete adjuvant	10	0·1
	35	0·01
	49	0·024
	10	0·21
	35	0·16
	49	0·07
40 μg HSA in water-in-oil emulsion	10	0·16
	35	0·032
	49	0·025
	10	0·23
	35	0·18
	49	0·11
40 μg HSA in 0·15 M saline	10	0·19
	35	0·2

concentrations which correspond with an ABC_{20} of 50 per cent of the ABC_{20} at the highest antigen concentration (1·0 μg/ml). The values obtained were accepted as providing a quantitative assessment of antibody avidity, sera of *high* avidity achieving the 50 per cent point at a *low* antigen concentration. It can be seen that sera obtained after injection of antigen in 0·15 M NaCl are of relatively low avidity (0·19 and 0·2 μg/ml at 10 and 35 days after injection). The avidity of the 10-day sera from birds injected with either Freund's complete or incomplete adjuvant mixture are of the same order (0·1, 0·21, 0·16 and 0·23 μg/ml) as those from birds injected with antigen in saline. After this, at the 35th and 49th days the avidity increased with both adjuvant procedures, sometimes greatly so; by the 49th day values were obtained of 0·024 and 0·07 μg/ml (Freund's complete adjuvant) and

0·025 and 0·11 μg/ml (water-in-oil adjuvant). No determinations were made on sera obtained 49 days after injection with antigen in saline as no antibody was detected in these sera.

CELLULAR PRODUCTION OF ANTI-HSA BY VARIOUS TISSUES FOLLOWING INJECTION OF 40 μg IN HSA IN 0·15 M NaCl, FREUND'S INCOMPLETE AND COMPLETE ADJUVANT

Sites of antibody production during the second phase of anti-HSA production (21st day onwards) following intramuscular injection of 40 μg HSA in Freund's complete adjuvant, water-in-oil emulsion or 0·15 M NaCl were investigated using the fluorescent antibody technique to demonstrate the presence of anti-HSA. Tissues were removed from ten birds 40–56 days, after injection (Table 5). Spleens only were examined from birds killed on the 40th, 46th and 56th days. Tissues examined from the three birds killed on the 54th day are listed in Table 6. No anti-HSA containing plasma cells were detected in any of the sections examined despite high circulating antibody levels. In particular no plasma cells containing anti-HSA were seen in the red pulp of the spleen. However, in sections from six out of ten birds examined anti-HSA containing cells were seen in a small proportion of the germinal centres. The number of such cells in the section of any one centre varied from one to eight. Morphologically these cells resembled in outline medium lymphocytes showing a centrally placed nucleus with a rim of brightly fluorescent cytoplasm (Fig. 1). Approximately half the birds from each of the three types of injection procedure

TABLE 5

NUMBERS OF BIRDS WHOSE TISSUES WERE INVESTIGATED BY IMMUNOFLUORESCENCE AT VARIOUS TIMES AFTER A SINGLE INJECTION OF HSA

Injection procedure	Days after HSA injection			
	40	46	54	56
40 μg HSA intramuscularly in Freunds' complete adjuvant	1	1	3	1
40 μg HSA in water-oil (Freund's incomplete adjuvant)	1	1	–	–
40 μg HSA in 0·15 M NaCl	1	1	–	–

TABLE 6

ANTIBODY CONTENT OF TISSUE EXTRACTS OBTAINED FROM CHICKENS 54 DAYS AFTER INJECTION OF 40 μg HSA IN FREUND'S COMPLETE ADJUVANT

	Antibody content (ABC_{30})		
	Chicken 143	Chicken 145	Chicken 146
Tissue (μg/g)			
Granuloma	132·0	1200·0	120·0
Caecum	28·0	17·0	1·62
Bursa	8·3	20·8	–
Spleen	6·7	11·8	2·95
Lung	26·7	–	–
Liver	20·8	19·0	–
Thymus	–	14·4	5·1
Skin	–	41·8	23·8
Bone marrow	–	17·0	15·2
Serum (μg/ml)	48·0	780·0	21·0

showed anti-HSA in the cells of their germinal centres. For technical reasons examination by the fluorescent antibody technique of sections of bone marrow and granulomata was not satisfactory, in the first case due to the presence of large numbers of naturally and non-specifically fluorescent cells of the granulocyte series, and in the second case to a high specific background fluorescence produced by HSA in the granuloma.

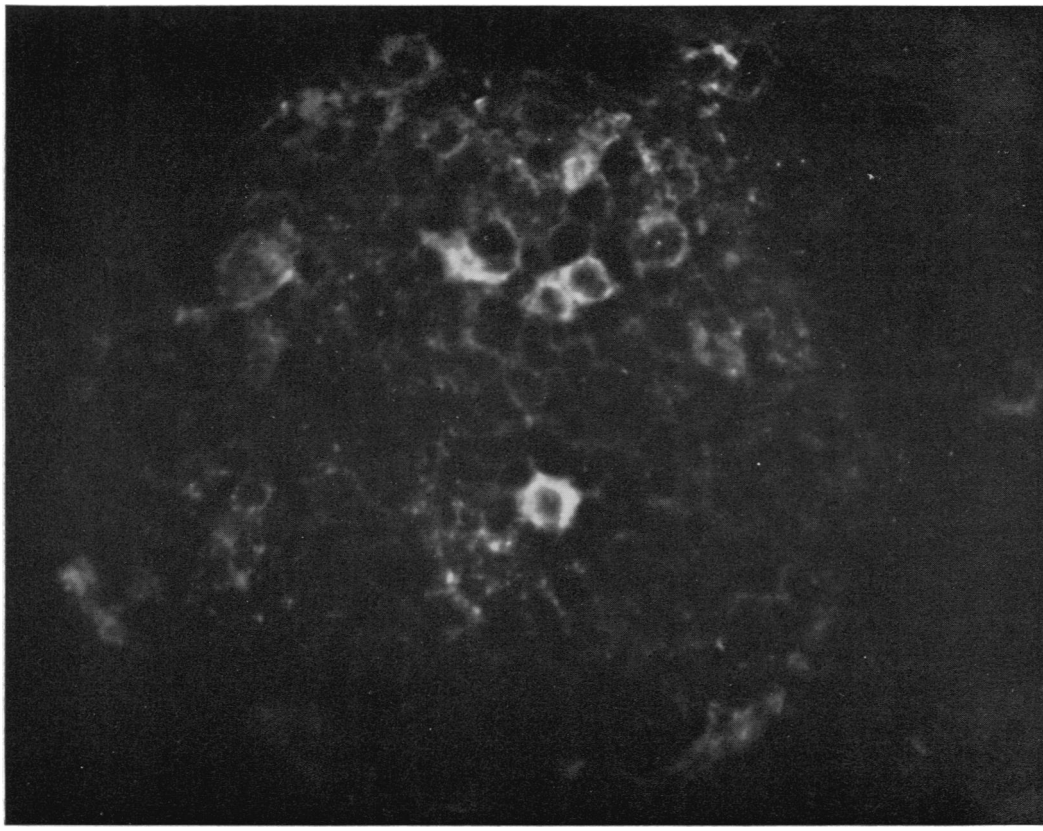

FIG. 1. Fluorescence micrograph of spleen of chicken obtained 40 days after intramuscular injection of 40 μg HSA in Freund's complete adjuvant. The section has been stained by the 'sandwich' technique to demonstrate anti-HSA. Six medium lymphocytes with anti-HSA in their cytoplasm are present. × 1040.

ANTI-HSA CONTENT OF TISSUES

The antigen (HSA)-binding capacity of tissue extracts prepared from liver, spleen, bursa, thymus, lung, skin, bone marrow, caecal tonsil and granulomatous tissue was measured as detailed in 'Materials and methods'. The results are given in Table 6. These show that the HSA-binding capacity per unit weight of the tissue extract obtained from the granuloma formed at the site of injection of 40 μg HSA in Freund's complete adjuvant was at least four times greater than that of extracts from other tissues including the spleen and bone marrow. The ABC_{30} per gram of granuloma was between twice and six times greater than the ABC_{30}/ml serum taken from the bird at the time of death.

DISCUSSION

As shown in Tables 2 and 3 the injection of HSA in water-in-oil emulsion with or without mycobacteria results in a biphasic serum antibody response. The initial phase of this (up to the first peak) is little different from that resulting from a single intravenous injection of the same amount of HSA in 0·15 M NaCl (Steinberg *et al.*, 1970). The prolonged secondary rise of antibody production caused by the intramuscular injection of HSA in water-in-oil emulsion begins about the 21st day and is still in evidence as late as 59 days after injection. An even greater increase in antibody production in the second phase is observed when *M. tuberculosis* is added to HSA in a water-in-oil emulsion. The HSA injected in emulsion provokes a second peak of antibody production presumably because the slowly released antigen remains available to stimulate a secondary response.

The results of avidity-testing indicate that both adjuvant procedures, antigen in water-in-oil emulsion with or without mycobacteria, greatly influence the properties as well as the amount of antibody produced in the late phase of the response. The highest avidities were detected in the sera of birds given antigen in Freund's complete adjuvant (at the 35th day in one bird, at the 49th day in the others). Antibody of increased avidity is found in the later bleedings from animals injected with small amounts of antigen (Eisen and Siskind, 1964) or after repeated injections of antigen. The depôt effect of the adjuvant mixtures would reproduce such conditions by allowing the escape of small amounts of antigen over a prolonged period. It is clear that antibody of progressively increasing avidity is produced by the chicken injected with antigen in Freund's incomplete adjuvant. The same is true of Freund's complete adjuvant but the data are inadequate to show whether the addition of mycobacteria accelerates or enhances this process.

In contrast to findings during the first phase of antibody production, anti-HSA containing plasma cells were not found in the splenic red pulp nor in any other tissue during the second phase of antibody production in birds injected with HSA in adjuvant, despite high circulating anti-HSA levels. However, a few medium lymphocyte cells containing anti-HSA were present in some of the germinal centres of the chicken spleen. Compared with the plasma cells present in the red pulp during the initial phase of the antibody response, these cells were few in number and unlikely to be the main source of circulating anti-HSA during the second phase of antibody production. Their presence indicates that the germinal centres are active for at least 1–2 months after a single injection of antigen in complete adjuvant and it is possible that the primed cells from this source migrate to become antibody-producing cells in the granuloma.

Conventional histological examination of the granulomata produced by HSA in Freund's complete adjuvant showed an intense infiltration of plasma cells around the periphery of the granuloma: these cells may be the source of the anti-HSA produced during the second phase. This is supported by the high anti-HSA content of the granuloma extracts, between four and thirty times greater than that of other tissues (including the spleen and bone marrow). This compares with the large amount of specific antibody synthesized in adjuvant granulomata of mammalian species such as the horse (Freund, Schryver, McGuinness and Geitner, 1952) and rabbit (Askonas and Humphrey, 1958), and contrasts with the guinea-pig in which the contribution of the adjuvant granuloma to antibody production is small (Askonas and White, 1956).

From our findings anti-HSA seems unlikely to be present in the granulomata largely as the result of non-specific inflammation since, in all instances, the serum level (ABC_{30}/ml)

was appreciably lower than that in a comparable weight of granuloma (ABC_{30}/g). Anti-HSA may be present in the granuloma in the form of antigen–antibody complexes which might dissociate during the process of tissue extraction and be detected in the Farr test. However, since only 40 μg HSA was injected the amount of HSA held in the granulomata must be very small. Also, the HSA-binding capacity of the granulomata tissue extracts (120–1200 μg/ml) far exceeded that required to bind all the injected HSA. This, taken with the histological findings, suggests that the high anti-HSA content in the granuloma is best explained by production of anti-HSA in the granuloma in quite large amounts.

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