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Antigen Uptake *In Vivo* by Peritoneal Macrophages from Normal Mice, and those undergoing Primary or Secondary Responses

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Summary. The *in vivo* uptake of human serum albumin and ferritin by peritoneal macrophages from normal, primary and secondary response mice has been studied. Both antigens were ingested by the macrophages irrespective of whether they were injected simultaneously or sequentially. Less than 1 per cent of the cells ingested only one antigen.

There was no indication that one antigen was ingested in preference to the other in any of the groups of mice.

The significance of these findings for the immune response is discussed.

INTRODUCTION

Various reports in the literature indicate that some particulate antigens are taken up in preference to others by the reticulo-endothelial system. Thus Halpern, Biozzi, Benacerraf and Stiffel (1957) showed that carbon particles are phagocytosed by guinea-pig reticulo-endothelial cells in preference to pigeon erythrocytes *in vivo*; albumin–globulin complexes are taken up more readily than carbon particles by the same type of cells (Benacerraf, Biozzi, Halpern and Stiffel, 1957). Experiments carried out by Perkins and Leonard (1963) indicated that mouse peritoneal cells selectively engulf erythrocytes from different species.

It is still an open question as to whether a preferential uptake of soluble antigens occurs; this might be of significance for the immunological response, since the simultaneous or sequential injection of different soluble antigens can cause a depression of antibody response to one of them (Adler, 1964). Adler (1964) suggested that such a competition might occur in the uptake and utilization of antigen.

In our experiments two soluble antigens, human serum albumin (HSA) and ferritin, were labelled with different fluorochromes, a technique which has already been described by Litt (1964) and Cebra and Goldstein (1965). Heat aggregates of the same conjugates were used as particulate antigens. The *in vivo* uptake of these antigens by peritoneal macrophages from normal, primary and secondary response mice was investigated.

MATERIALS AND METHODS

Animals

Two batches of inbred C3H mice of both sexes, bred at the Statens Seruminstitut, were immunized according to the following schedule:

IMMUNIZATION SCHEDULE FOR C3H MICE

Group	Primary stimulus 2 months old*	Secondary stimulus 4 months old*
Secondary response mice		
1A	HSA	HSA
1B	HSA	Ferritin
1C	HSA	HSA + ferritin
Controls 1	HSA	—
2A	Ferritin	HSA
2B	Ferritin	Ferritin
2C	Ferritin	HSA + ferritin
Controls 2	Ferritin	—
Primary response mice, 4 months old		
3	HSA	—
4	Ferritin	—
5	HSA + ferritin	—
6	—	—
Normal mice, 4 months old		

Each group = ten mice. — = Nothing given. The mice were used 12 weeks after the last injection.

* 100 μg alum-precipitated antigen in hind footpads.

In addition, 2-month-old normal mice were injected with 100 μg alum-precipitated antigen into the hind footpads, corresponding to Groups 3, 4, and 5 in the above schedule. These mice were used 8 weeks after injection.

Haemagglutination

The antibody response to HSA and ferritin was determined by means of a modification of Boyden's passive haemagglutination technique using formalinized sheep erythrocytes (Stavitsky, 1964).

Conjugation of ferritin and HSA with the isothiocyanates of fluorescein and tetramethylrhodamine

Ferritin (cadmium-free) was obtained from Pentex, Illinois. Human serum albumin (HSA) was supplied by Miss K. Østergaard, Blood Fractionation Department, Statens Seruminstitut. It had been prepared according to a modified Cohn technique (Fraction V). Fluorescein isothiocyanate (FITC) Lot No. 302699 and Lot No. 307649 and tetramethylrhodamine isothiocyanate (TMRITC) Lot No. 401671 were obtained from Baltimore Biological Laboratories.

The conjugation with FITC was performed according to Marshall, Eveland and Smith (1958) and that with TMRITC according to Cebra and Goldstein (1965). Each batch of the conjugates was prepared as follows: For the labelling of ferritin with FITC, 7 ml of physiological saline and 1 ml 0·5 M carbonate–bicarbonate buffer, pH 9·0, were added to 2 ml of a 10 per cent solution of ferritin (95–105 mg/ml). Crystalline FITC (10 mg) was then added. For labelling with TMRITC, 200 mg HSA was dissolved in 9 ml physiological saline and mixed with 1 ml 0·5 M carbonate–bicarbonate buffer, pH 9·0. Crystalline TMRITC (10 mg) was then added. The mixtures were stirred overnight at 4° by means of magnetic stirrers. Surplus dye was removed by filtration through Sephadex G-25 for ferritin–FITC, and Sephadex G-50 for HSA–TMRITC.

The content of dye and protein in each conjugate was determined using a Beckman Spectrophotometer DU. The absorption due to FITC was measured at 495 mμ and that of TMRITC at 500 mμ. At these wavelengths the absorption due to protein is negligible and, therefore, the content of dye within the conjugates can be read directly from a standard curve representing the correlation between optical density (O.D.) and concentration of the dye. When measuring for protein content at 280 mμ, it was necessary to correct for the absorption due to the dyes at this wavelength (Tokumara, 1962). The correlation between the O.D. readings at 280 and 495 mμ (500 mμ) at various concentrations of the pure dyes was depicted in two curves. The O.D. at 280 mμ corresponding to a measured O.D. value for the conjugate at 495 mμ (500 mμ) was read from this curve and subtracted from the O.D. value of the conjugate measured at 280 mμ. The protein content of the conjugate was calculated from this corrected value for the O.D. at 280 mμ.

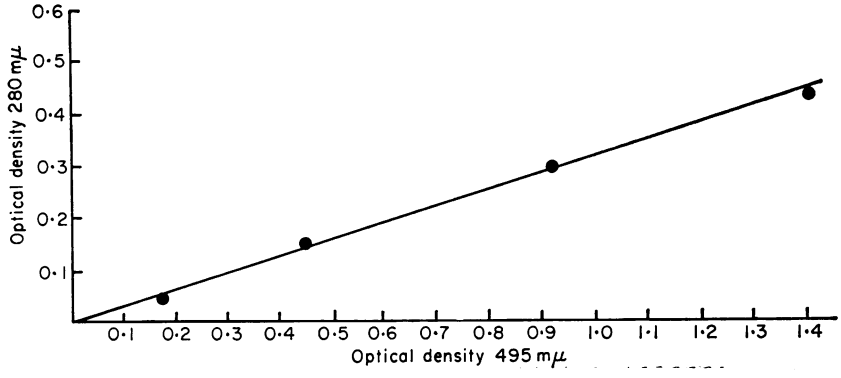


FIG. 1. Correlation between optical density of FITC at 280 mμ and 495 mμ at various concentrations between 0·1 and 10 μg FITC/ml.

The stability of the conjugates under the present experimental conditions was evaluated by heating to 37° for 1, 2, 4, and 6 hours. The antigens were then precipitated with their specific antisera. After being washed four times the antigen–antibody complexes were examined for changes in colour or in degree of fluorescence. The conjugates proved to be stable after heating at 37°.

The conjugates gave only one line of precipitation against the appropriate specific antiserum after immunoelectrophoretic separation. The electrophoretic mobilities were greater than for unconjugated antigens.

The conjugates were stored at −20°, but normally not for more than 14 days since the intensity of the fluorescence tended to diminish after longer storage.

Before injection into mice, the soluble conjugates were centrifuged at 12,000 ***g*** for 10 minutes and sterilized by passage through a Millipore filter.

Preparation of particulate antigens

The soluble conjugates were first sterilized by passage through a Millipore filter, then aggregated by heating at 70° for 20 minutes at pH 5·0, and finally restored to pH 7·0.

Procedure for the uptake of antigen

In order to obtain a high yield of macrophages, mice were injected intraperitoneally with 2 ml of 10 per cent proteose–peptone in saline. After 3 days the mice were injected

with the fluorescent antigens. On an average each mouse contained a total of approximately 8×10^6–10^7 peritoneal exudate cells.

(a) *Preliminary experiments with normal and primary response mice.* Normal mice were injected with a quantity of soluble fluorescent antigen corresponding to 100 μg, 1, 2 and 6 mg protein. The cells were harvested from the peritoneal cavity at 1, 2, 4 and 24 hours after injection and treated as described below.

Heat-aggregated antigens in 3-mg quantities were injected and harvested from the peritoneal cavity at the same intervals as the soluble antigens. This dose was used because 6 mg of aggregated protein overloaded the cells, and resulted in much extracellular non-phagocytosed material.

Simultaneous injection of two antigens. Ten mice were subdivided into groups of two mice; these subgroups were injected intraperitoneally with antigen(s) according to the following schedule:

Group 1: 6 mg HSA–TMRITC.
Group 2: 6 mg ferritin–FITC.
Group 3: 6 mg HSA–TMRITC followed immediately by 6 mg ferritin–FITC.
Group 4: 6 mg ferritin–FITC followed immediately by HSA–TMRITC.
Group 5: Controls.

Three milligrams only of the particulate antigens were injected as above.

Sequential injection of two soluble antigens. An interval of 10 minutes, 30 minutes, 1 hour or 2 hours between the injection of the first and second antigens was included in the experiments, otherwise the procedure was identical to that described above.

(b) *Experiments with mice immunized according to the schedule* (*p.* 512). One batch of mice was used for injection of soluble antigens and the other for injection of particulate antigens.

Each group of ten mice (normal, primary and secondary response mice) was subdivided into groups of two mice and injected as follows:

Group 1: 6 mg HSA–TMRITC.
Group 2: 6 mg ferritin–FITC.
Group 3: 6 mg HSA–TMRITC or ferritin–FITC followed immediately by ferritin–FITC or HSA–TMRITC.
Group 4: 6 mg HSA–TMRITC or ferritin–FITC, 1 hour's interval, injection of ferritin–FITC or HSA–TMRITC.
Group 5: Controls.

In the subgroups 3 and 4 the antigen which had been used for the primary stimulus in the immunization schedule was injected first.

The mice were killed 4 hours after injection of antigen and the cells were harvested by washing out the peritoneal cavity with 3 ml saline containing heparin 1:20,000 and 5 per cent normal rabbit serum. The cells were centrifuged down at 700 ***g*** for 3 minutes and then washed twice in Gey's solution and once in saline. The cells were resuspended in saline and adjusted to a density of about 10^6 cells/ml. One-tenth of a millilitre was spun down onto a microscope slide using a spreading device (Doré and Balfour, 1965). The smears were examined unfixed.

Procedure for the elimination of antigen

Soluble antigen corresponding to 6 mg protein and aggregated antigen corresponding

to 3 mg protein were injected intraperitoneally into normal and primary response mice. Peritoneal exudates were collected 1, 2, 4 and 24 hours after injection. After centrifugation the O.D. of the supernatants were measured at 495 and 500 mμ, respectively. The dye content was read from a standard curve as described before.

Microscopic examination of the cells

Fluorescence microscopy. The microscope employed was a Zeiss Universal Microscope fitted with an ultra darkfield condenser and oil immersion objectives ('apo 40 m.I.' and 'planapo 100 m.I.'). The light source was an Osram HBO 200 high pressure mercury lamp. The exciter filters were BG 38 (built into the microscope) and BG 12 (3 mm). The barrier filters were Zeiss Nos. 47 or 50.

Examination of the cells was carried out not later than 1 day after preparation of the smears, in order to avoid autofluorescence of the cells and because the intensity of the specific fluorescence decreased to some extent with time. A count of 400–500 cells was made for each slide and differentiated as follows: (a) total number of cells ingesting antigen, (b) number of cells ingesting one antigen and not the other, and (c) total number of cells. Areas were avoided where there was clumping of cells.

Differential cell counts. Cell counts were performed using ordinary light microscopy on the same smears cleaned in xylene and stained with Wright's stain. The percentage of the different types of cells present was determined. The percentage of macrophages ingesting antigen(s) was calculated from these data and those obtained by fluorescence microscopy.

Photomicrography

The equipment consisted of a Zeiss Attachment Camera with a basic body II. The films employed were Anscochrome and Ektachrome (highspeed, daylight). Exposures varied from 1 to 3 minutes for photomicrographs of cells examined by fluorescence microscopy. After photographing the fluorescent cells, the position of the slide was noted. The smear was then stained with Wright's stain, examined by ordinary light microscopy, and photographed in the same position using the same films and applying a conversion filter and appropriate neutral grey filters. All magnifications given are corrected for a camera-factor of 0·5.

RESULTS

ANTIBODY RESPONSE TO HSA AND FERRITIN

The levels of antibodies in mice which were immunized according to the schedule described in 'Material and methods' were measured by the passive haemagglutination test (Stavitsky, 1964). The results are shown in Tables 1 and 2.

The primary response to HSA (Table 1, Group 3) was slightly lower than that to ferritin (Table 2, Group 4). In Group 1B ferritin had a stimulatory effect on the production of antibody to HSA, which had been given as a primary stimulus 2 months previously. The titre was lower than that for the secondary response to HSA (Table 1, Groups 1A and 1C). No stimulatory effect of HSA was observed on the production of antibody to ferritin (Table 2, Group 2A).

There was no definite evidence of competition of antigen in the three groups in which this might have occurred, namely in Groups 1C, 2C and 5.

Group 2B (Table 1) showed a primary response to HSA, and the only explanation for this is that these mice were inadvertently injected with HSA and ferritin, as in Group 2C. In the other batch of mice immunized according to the same schedule there was no primary response to HSA in Group 2B.

TABLE 1

SERUM LEVELS OF ANTI-HSA IN NORMAL, PRIMARY AND SECONDARY RESPONSE MICE (TEN MICE PER GROUP), MEASURED BY THE PASSIVE HAEMAGGLUTINATION TEST, AND EXPRESSED AS $\log_{10}$ TITRE

Group	Titre before re-injection	Weeks after injection				
		2	4	6	8	12
1A	2·2 (30 mice)	4·4	4·1	3·9	3·7	3·3
1B		3·4	3·3	2·9	2·9	2·6
1C		4·4	4·0	3·9	3·8	3·6
2A	—	2·7	2·0	2·3	1·8	2·0
2B	—	2·8	2·0	2·2	2·2	1·8
2C	—	2·9	2·9	3·0	2·5	2·5
3	—	1·6	2·0	2·2	2·1	1·6
4	—	—	—	—	—	—
5	—	2·1	2·7	2·6	2·6	1·6
6	—	—	—	—	—	—

Standard error for one $\log_{10}$ titre = 0·0927.
The difference between two log titres is significant if this value is 0·2622.
— = No reaction.

TABLE 2

SERUM LEVELS OF ANTI-FERRITIN IN NORMAL, PRIMARY AND SECONDARY RESPONSE MICE (TEN MICE PER GROUP), MEASURED BY THE PASSIVE HAEMAGGLUTINATION TEST, AND EXPRESSED AS $\log_{10}$ TITRE

Group	Titre before re-injection	Weeks after injection				
		2	4	6	8	12
1A	—	—	—	—	—	—
1B	—	2·3	2·9	2·9	2·7	2·3
1C	—	2·2	2·6	2·6	2·2	1·9
2A	2·8 (30 mice)	2·1	2·0	1·9	1·9	1·1
2B		4·4	4·4	4·0	3·8	3·6
2C		3·4	3·4	3·6	3·5	3·3
3	—	—	—	—	—	—
4	—	3·1	3·1	3·2	3·1	2·6
5	—	2·2	2·6	2·6	2·9	2·6
6	—	—	—	—	—	—

Standard error for one $\log_{10}$ titre = 0·0927.
The difference between two log titres is significant if this value is 0·2622.
— = No reaction.

The antibody titres decreased with time, but a primary and secondary response were still evident when the mice were used 12 weeks after the last injection.

ELIMINATION OF SOLUBLE AND PARTICULATE ANTIGENS FROM THE PERITONEAL CAVITY

As shown in Figs. 2 and 3, about 95 per cent of both soluble and particulate antigen were eliminated after 4 hours in normal mice. The initial rate of elimination of soluble

antigen was considerably less than that for particulate antigen, a result which was only to be expected since particulate matter is ingested more rapidly than soluble by peritoneal cells (Rhodes, 1964).

In primary response mice the soluble conjugates were eliminated slightly more rapidly within the first 2 hours than in normal mice. Particulate antigen was eliminated at the same rate in primary response as in normal mice.

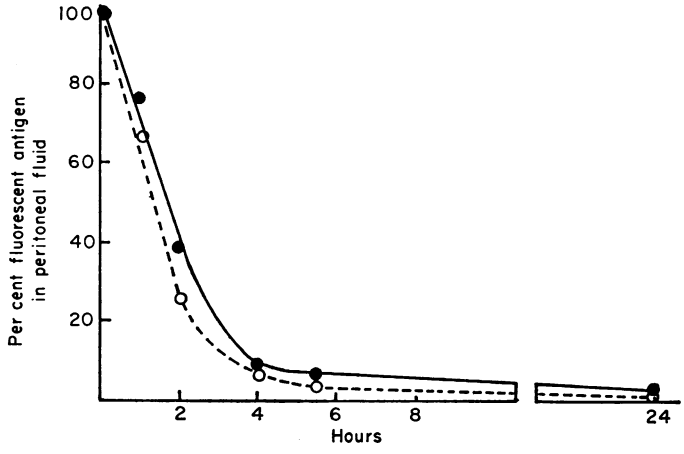


FIG. 2. Rate of elimination of soluble antigens in normal mice. ●, HSA–TMRITC; ○, ferritin–FITC.

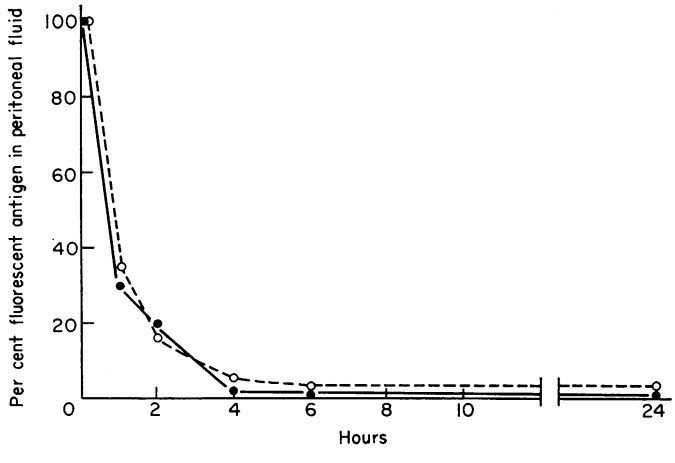


FIG. 3. Rate of elimination of particulate antigens in normal mice. ●, HSA–TMRITC; ○, ferritin–FITC.

THE UPTAKE OF ONE ANTIGEN

Soluble antigens

Examination of the exudate cells showed a change in the composition of the cell population with time. The intensity of the fluorescence of the ingested antigen was enhanced with increase in dosage and in time as regards the first 2–4 hours after injection. Based on these preliminary results we decided to inject 6 mg of antigen and to harvest the cells 4 hours after injection. Four hours was the most convenient interval both as

regards the intensity of fluorescence and the fact that polymorphonuclear cells (PMN) induced by the injection of antigen were then receding.

A list of the data pertaining to the uptake of soluble antigen in the exudate cells is given in Table 3, with the percentage of the various types of cells present at the different time intervals.

The majority of peritoneal macrophages from normal mice, which had been in contact with soluble antigen *in vivo* for 4 hours, ingested antigen as small droplets showing intense fluorescence. Pinocytic vesicles were seen scattered throughout the cytoplasm, but the fluorescence was not associated with these vesicles. No fluorescence was seen in the nucleus, unless the cells were damaged in some way; in this case the whole cell showed a diffuse fluorescence. PMN cells and eosinophils pinocytosed a very small amount of soluble material.

The fluorescence of ferritin–FITC was yellow-green, whilst that of HSA–TMRITC was orange. The droplets with orange fluorescence were normally more variable in size than those with yellow-green fluorescence (see Figs. 4 and 5). Ferritin–FITC sometimes showed a fluorescence ranging from pale-blue to yellow-green within the same cell. In the majority of the macrophages harvested 24 hours after injection the ferritin–FITC was seen as distinctly bluish droplets. Ingested, unlabelled ferritin was shown to have a strong blue fluorescence. Changes in colour were not observed for HSA–TMRITC.

That the cells ingesting antigens were macrophages and medium-sized mononuclear cells is demonstrated in Figs. 5 and 6. Fig. 5 shows yellow-green fluorescent droplets of ferritin–FITC in the cytoplasm. Fig. 6 illustrates the same cells after staining with

FIG. 4. Macrophage and medium-sized mononuclear cells from the peritoneal cavity of normal mice injected with 6 mg soluble HSA–TMRITC intraperitoneally. The cells were harvested 24 hours after injection. The fluorescence is in small droplets scattered throughout the cytoplasm, but not in the nucleus or the vacuoles. × 640, filter 50.

FIG. 5. Macrophage and medium-sized mononuclear cells from the peritoneal cavity of normal mice injected with 6 mg ferritin–FITC intraperitoneally. The cells were harvested 4 hours after injection. The yellow-green droplets are scattered throughout the cytoplasm, but not in the nucleus or vacuoles. × 1000, filter 47.

FIG. 6. The same cells as in Fig. 5 stained with Wright's stain. The large cell has the typical structure of a macrophage. The violet particles in the cytoplasm are ferritin and correspond to the green fluorescent droplets in Fig. 5. These particles are not visible in the pinocytic vesicles. Note that the two mononuclear cells on the left have not ingested the antigen. × 1000.

FIG. 7. Macrophages from the peritoneal cavity of mice, with a primary response to ferritin, injected with 3 mg aggregated HSA–TMRITC intraperitoneally. The cells were harvested 4 hours after injection. The cell in the left-hand bottom corner is a PMN cell. The large red clump at the top of the picture is extracellular aggregated HSA. × 640, filter 50.

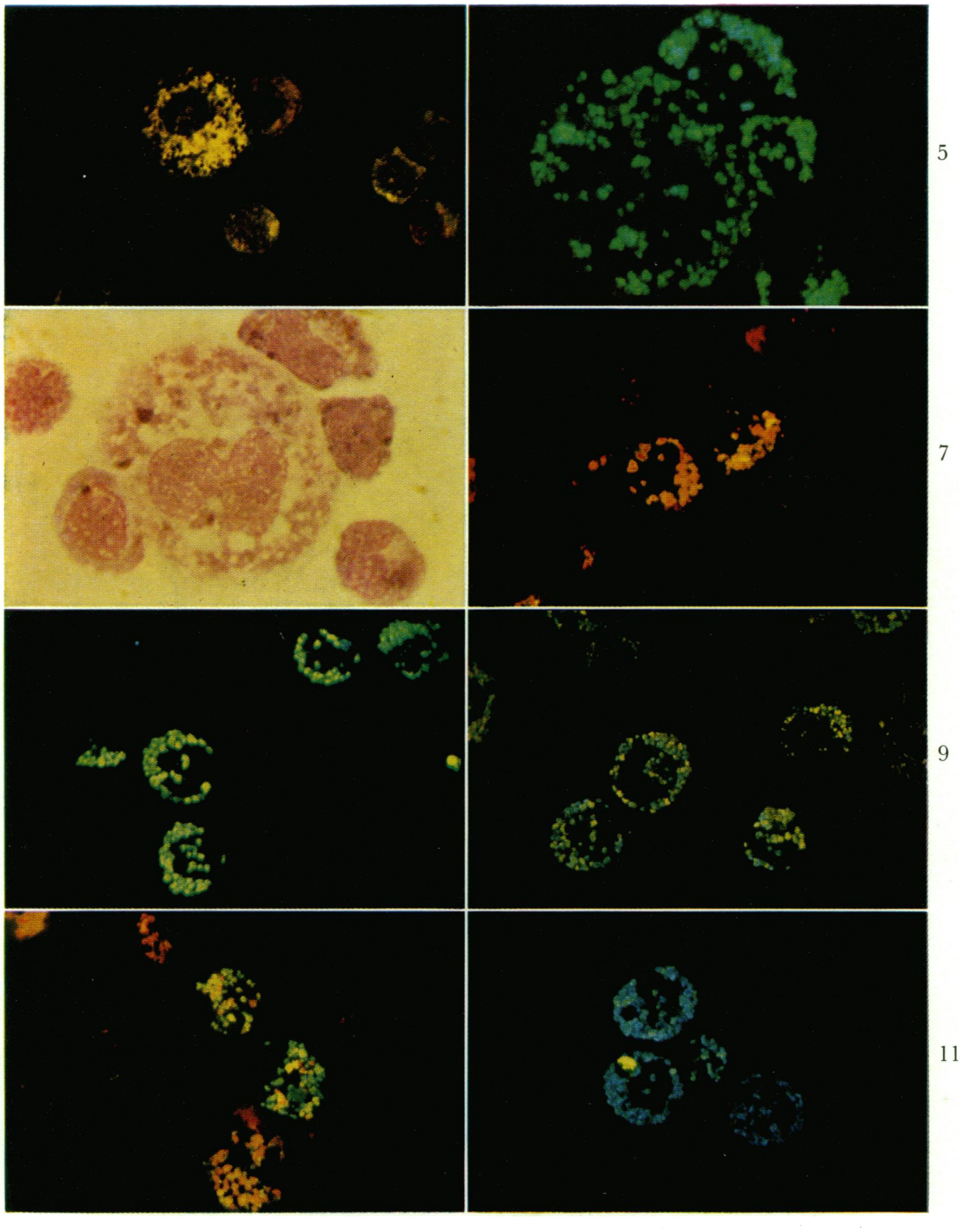
FIG. 8. Macrophages from the peritoneal cavity of mice, with a primary response to ferritin, injected with 3 mg aggregated ferritin–FITC intraperitoneally. The cells were harvested 4 hours after injection. × 640, filter 47.

FIG. 9. Macrophages from the peritoneal cavity of mice, with a primary response to ferritin, injected with 6 mg soluble HSA–TMRITC and 6 mg soluble ferritin–FITC intraperitoneally. The antigens were injected simultaneously. These cells were harvested 4 hours after injection. The two cells slightly to the left appear to contain more ferritin than HSA. × 400, filter 47.

FIG. 10. Macrophages from the peritoneal cavity of mice, with a primary response to ferritin, injected with 3 mg aggregated HSA–TMRITC and 3 mg aggregated ferritin–FITC intraperitoneally. There was an interval of 1 hour between injections. The cells were harvested 4 hours after injection. The cell at the top, slightly to the left is a PMN cell and contains only HSA–TMRITC. The macrophage in the middle obviously contains more ferritin than HSA, whilst the cell at the bottom contains more HSA than ferritin. The intense red clumps between the two cells at the bottom of the picture are extracellular aggregated HSA–TMRITC. × 640, filter 47.

FIG. 11. Macrophages from the peritoneal cavity of mice, with a primary response to ferritin, injected with 3 mg aggregated ferritin–FITC. The cells were harvested 24 hours after injection. Note that the colour of the aggregated ferritin–FITC has changed from yellow-green to blue, particularly in the macrophage on the right, which contains chiefly blue particles. × 640, filter 47.

Antigen Uptake In Vivo



(*Facing p.* 518)

TABLE 3

UPTAKE OF SOLUBLE HSA–TMRITC AND FERRITIN–FITC INTO NORMAL MOUSE PERITONEAL EXUDATE CELLS (MORPHOLOGICAL EVALUATION FROM FLUORESCENT MICROSCOPY AND LIGHT MICROSCOPY)

Dose injected	Hours after injection	Type of cells taking up antigen	Fluorescence		Cell counts (%)		
			Colour of droplets	Intensity	Macrophages	PMN	Mononuclear
HSA–TMRITC							
6 mg per mouse	1	Macro. + mono. cells	Orange	+	56	8	24
	2	Macro. + mono. cells	Orange	++	29	53	15
	4	Macro. + mono. cells	Orange	++	42	30	25
	24	Macrophages	Orange	++	66	8	23
Ferritin–FITC							
6 mg per mouse	1	Macro. + mono. cells	Yellow-green	+	58	12	22
	2	Macro. + mono. cells	Yellow-green	++	11	53	22
	4	Macro. + mono. cells	Yellow-green	++	42	42	13
	24	Macrophages	Yellow-green and blue	++	70	2	23
Control cells, 3-day exudate					80	—	15

+ = Rather strong fluorescence; ++ = Intense fluorescence. Macrophages = Large vacuolated mononuclear cells; PMN = polymorphonuclear cells; Mono. = medium and small mononuclear cells, excluding small lymphocytes.

Wright's stain. The cell in the centre has the typical structure of a large macrophage with many pinocytotic vacuoles in the cytoplasm. The small dark particles scattered throughout the cytoplasm are ferritin and correspond to the fluorescent droplets in Fig. 5. This plate illustrates the fact that some medium-sized mononuclear cells do not ingest antigen.

Macrophages from primary and secondary response mice of all groups showed similar patterns of uptake of a single soluble antigen, to those found for normal mice.

Particulate antigens

The injection of particulate antigen into normal mice induced a cell population which was completely different from that obtained after the injection of soluble antigen (see Table 4).

At 4 hours there were hardly any large macrophages, but about 60–70 per cent PMN and about 10 per cent mononuclear cells. In contrast to the discrete droplets of ingested soluble antigen, these macrophages contained large particles of aggregated antigen (see Figs. 7 and 8). The fluorescence of aggregated HSA–TMRITC was red with a tendency towards orange at the periphery of the particles. Ferritin–FITC fluoresced yellow-green both in the soluble and the aggregated state, the fluorescence of the latter being more brilliant. The fluorescence of the two antigens had reached its maximum intensity in the cells one hour after injection.

PMN readily engulfed the aggregated antigens, in particular HSA–TMRITC (see Fig. 7).

Macrophages harvested from primary and secondary response mice showed the same pattern of phagocytosis of the particulate antigens as that found in normal mice.

THE SIMULTANEOUS AND SEQUENTIAL UPTAKE OF TWO ANTIGENS

Soluble antigens

Results from an experiment using normal mice injected simultaneously with two soluble antigens are illustrated in Table 5. Cells from Group 1 showed an orange fluorescence due to HSA–TMRITC, and those in Group 2 a yellow-green fluorescence due to ferritin–FITC. Cells from Groups 3 and 4 contained bluish, yellow-green, and orange droplets suggesting the presence of two antigens (see Fig. 9). Less than 1 per cent of the cells contained only HSA or ferritin.

The percentage of macrophages ingesting antigen was calculated from: (a) the percentage of large macrophages present in the peritoneal exudate estimated by a differential count, and (b) the number of large macrophages in a given population of cells having ingested antigen. Seventy to 80 per cent of the large macrophages took up antigens. This figure was only slightly lower when medium-sized mononuclear cells were included.

Injection of the second antigen after an interval of 10 minutes, 30 minutes, 1 hour or 2 hours had no influence on the uptake or distribution of either of the antigens.

Macrophages from primary and secondary response mice ingested the two antigens in the same way as normal mice.

Aggregated antigens

The majority of macrophages contained both antigens, the distribution of particulate antigens resembling that found for soluble antigens (see Fig. 10). PMN cells tended to phagocytose HSA and only a few ingested ferritin; a mixed uptake was not seen in these cells.

TABLE 4

UPTAKE OF PARTICULATE HSA–TMRITC AND FERRITIN–FITC INTO NORMAL MOUSE PERITONEAL EXUDATE CELLS (MORPHOLOGICAL EVALUATION FROM FLUORESCENCE MICROSCOPY AND LIGHT MICROSCOPY)

Dose injected	Hours after injection	Type of cells taking up antigen	Fluorescence		Cell counts (%)		
			Colour of droplets	Intensity	Macrophages	PMN	Mononuclear
HSA–TMRITC 3 mg per mouse	1	Macrophages + PMN	Red	++	12	17	42
	2	Macrophages + PMN	Red	++	13	60	22
	4	Macrophages + PMN	Red	++	6	57	30
	24	Macrophages + PMN	Red	++	40	19	30
Ferritin–FITC 3 mg per mouse	1	Macrophages + PMN	Yellow-green	++	12	39	34
	2	Macrophages + PMN	Yellow-green	++	10	76	12
	4	Macrophages + PMN	Yellow-green	++	6	80	14
	24	Macrophages + PMN	Yellow-green	++	35	58	3

+ = Rather strong fluorescence; ++ = intense fluorescence.

Macrophages = Large vacuolated mononuclear cells; PMN = polymorphonuclear cells; Mononuclear = medium and small cells, excluding small lymphocytes.

About 50–60 per cent of the large macrophages phagocytosed particulate antigens (see Table 6). Inclusion of medium-sized mononuclear cells caused this figure to drop from 60 to about 20 per cent.

The behaviour of macrophages from primary and secondary response mice was in all respects similar to that of macrophages from normal mice.

TABLE 5

UPTAKE OF SOLUBLE FERRITIN–FITC AND HSA–TMRITC INTO NORMAL MOUSE PERITONEAL MACROPHAGES 4 HOURS AFTER SIMULTANEOUS INJECTION

Group	Antigen injected i.p. 6 mg of each/mouse	Cell counts (%)			Macrophages taking up antigen (%)*		
		Macro.	PMN	Mono.	HSA	Ferritin	Both antigens
1	HSA–TMRITC	47	35	13	78	—	—
2	Ferritin–FITC	52	43	5	—	78	—
3	HSA–TMRITC, followed immediately by ferritin–FITC	39	53	8	<1	—	77
4	Ferritin–FITC, followed immediately by HSA–TMRITC	38	53	9	<1	—	77
5	Control 3-day exudate cells	76	—	16			

* Calculated from the percentage of large macrophages present in the peritoneal exudate and the number of large macrophages in a given population of cells having ingested antigen.

TABLE 6

UPTAKE OF PARTICULATE FERRITIN–FITC AND HSA–TMRITC INTO NORMAL MOUSE PERITONEAL MACROPHAGES 4 HOURS AFTER SIMULTANEOUS INJECTION

Group	Antigen injected i.p. 3 mg of each/mouse	Cell counts (%)			Macrophages taking up antigen (%)*		
		Macro.	PMN	Mono.	HSA	Ferritin	Both antigens
1	HSA–TMRITC	20	29	33	65	—	—
2	Ferritin–FITC	31	45	9	—	64	—
3	HSA–TMRITC, followed immediately by ferritin–FITC	9	60	22	—	—	62
4	Ferritin–FITC, followed immediately by HSA–TMRITC	10	49	23	—	—	50
5	Control 3-day exudate cells	80	—	15			

* See Table 5.

DISCUSSION

One hour after the intraperitoneal injection of soluble HSA–TMRITC or ferritin–FITC into normal mice, small discrete fluorescent droplets of the conjugate were visible in the cytoplasm of peritoneal macrophages. FITC and TMRITC injected alone, in the same dose as that present in the conjugate, were not ingested by the macrophages, but caused a weak diffuse staining of the whole cell. The droplets of fluorescent material seen in macrophages after the injection of the antigens are, therefore, a true indication that the antigens had been pinocytosed. Ferritin–FITC frequently showed a fluorescence

ranging from pale blue-green to yellow-green. Unlabelled ferritin possessed a blue fluorescence when examined by fluorescence microscopy. Thus, this colour range may be an expression of the varying amounts of FITC on the ferritin molecules.

Soluble antigen was ingested by 70–80 per cent of the large macrophages. This value was constant when medium-sized mononuclear cells were included for the calculation of the percentage cells which had ingested antigen. Only about 50–60 per cent of the large macrophages ingested particulate antigen. This value dropped to 20 per cent if medium-sized mononuclear cells were included, indicating that these cells do not phagocytose particulate antigen. Because there was only a small percentage (6–10 per cent) of macrophages present after injection of particulate antigen, the error in the calculation will be large. On the other hand, the value of 50–60 per cent is in agreement with that found by Lee and Cooper (1966) for the percentage of normal mouse peritoneal cells which actively phagocytose.

There appeared to be a correlation between rate of elimination and uptake of the antigens. After intraperitoneal injection 95 per cent of both conjugates was eliminated at 4 hours. However, the rate of uptake could not be determined quantitatively. Soluble conjugates were eliminated more rapidly (within 2 hours) in the primary response mice, presumably due to the formation of antigen–antibody complexes. Such complexes would be expected to be removed more rapidly than soluble antigen. The same result was recorded for the removal of soluble protein and complexes from the bloodstream (Talmage, Dixon, Bukantz and Dammin, 1951).

It seems clear from the present experiments that two antigens were engulfed concurrently by the same peritoneal cells from normal, primary and secondary response mice. This occurred whether the antigens were soluble or aggregated, or whether they were injected simultaneously or sequentially. In addition, experiments were carried out in which normal mice were injected after various intervals of time with a soluble followed by a particulate antigen, and *vice versa*. The same result was obtained uniformly.

Less than 1 per cent of the macrophages ingested only one soluble antigen, and when this did occur it was usually HSA which was engulfed. No macrophages were observed that had only ingested one particulate antigen, undoubtedly owing to the paucity of these cells in the exudate at 4 hours after injection.

Perkins and Leonard (1963) found that macrophages selectively phagocytose erythrocytes from different species of animals. Only a few cells contained two types of erythrocytes, whilst many contained either one or the other. The majority of macrophages under our experimental conditions ingested both antigens, whether soluble or particulate.

Unfortunately, we were unable to demonstrate serological evidence of antigenic competition in the present system, probably because a higher dosage of one of the antigens is required (Ben-Efraim and Liacopolous, 1965). Cells from mice that show this phenomenon might provide further information on the uptake of two antigens.

Various authors have put forward the hypothesis that macrophages play no essential role in the 'competition of antigen' (Scheckter and Sela, 1965; Stiffel, Ben-Efraim, Perramant and Laicopolous, 1966; Amkraut, Garvey and Campbell, 1966). These authors contend that the competition occurs during the phase of recognition of antigen by the immunologically competent cells. On the other hand, one cannot completely exclude the possibility that a selection could take place intracellularly. Uhr and Weissman (1965) presented evidence that a necessary step in the synthesis of antibody is the degradation of antigen by the lysosomes in the granules of the reticulo-endothelial cells.

If two antigens are degraded at different rates within lysosomes, then it is feasible that one antigen or antigenic determinant might induce the formation of specific antibody before the other. In the present experiments, there was a definite change in the fluorescence of the ferritin–FITC 24 hours after injection (Fig. 11). Most of the droplets possessed a bright blue fluorescence as against the yellow-green fluorescence seen at 1–2 hours after injection. This suggests that the ferritin might be degraded. No such alteration in fluorescence was observed with HSA–TMRITC after 24 hours.

In our experiments, the two antigens which were taken up by a single macrophage appeared to be situated on different lysosomes. The localization of the antigens in macrophages is being investigated further using ferritin and radioactive HSA, with subsequent examination of the cells with the electron microscope.

Another obscure point is the role of cytophilic antibody in the uptake of two antigens. Antibody cytophilic for macrophages has been shown by Nelson and Boyden (1967) and Berken and Benacerraf (1966) to be primarily responsible for the phenomenon of opsonization. In the view of this an attempt was made to demonstrate the presence of cytophilic antibody by flooding unfixed cell smears with fluorescent antigen. Neither this procedure nor the original radioactive method for detecting this antibody (Boyden and Sorkin, 1960) was positive. Other workers (Nelson and Boyden, 1967) have also failed to detect cytophilic antibody on macrophages using these procedures, but this does not exclude its presence.

If cytophilic antibody is necessary for the ingestion of antigen, then one must postulate the presence of natural antibodies to HSA and ferritin in normal mice, since there was no difference in the uptake of antigen in normal and immune mice.

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