

THE IMMUNOGENICITY OF ANTIGEN BOUND TO THE
PLASMA MEMBRANE OF MACROPHAGES*

BY EMIL R. UNANUE,† M.D., AND JEAN-CHARLES CEROTTINI,§ M.D.

(From the Department of Experimental Pathology, Scripps Clinic and Research
Foundation, La Jolla, California 92037)

(Received for publication 24 November 1969)

Knowledge of the fate of immunogenic molecules as well as of their interaction with lymphoid cells is essential for an understanding of the inductive phase of the immune response. Most antigens, be they soluble or particulate, can be taken up by macrophages of antibody-forming organs. This uptake of antigens by macrophages may be an important step in the induction and maintenance of an immune state as surmised by a variety of experimental observations (reviewed in reference 1). So far, most of the information on the fate and immunogenicity of antigen in macrophages has been obtained by employing peritoneal macrophages. A variety of antigens bound to live peritoneal macrophages when transferred to syngeneic recipients initiate an immune response (2-5). The nature, amount, and site in the cell of the immunogenic moiety of antigen in spite of considerable study are still uncertain. A series of investigations using extracts of macrophages containing phagocytosed antigens identified a highly immunogenic RNA (6) which was complexed to a small amount of antigen (7-9). This has led to the postulation that "processing" of antigens by the macrophages is an essential step in the immune response. However, whether or not such an RNA-antigen complex plays an important role in ordinary immune responses still needs to be determined (10, 11).

We previously observed that when soluble antigens like hemocyanin are taken up by macrophages, the major portion (80-95%) was rapidly catabolized and eliminated from the cell, while a small amount was retained for long periods (4, 5). Approximately two-thirds of the retained antigen was inside the cell apparently in lysosomes and one-third was bound to the plasma membrane (12). Some of the retained antigen presumably comprised the immunogenic moiety. The present report is a further characterization of the antigen bound to the

* This is publication number 384 from the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037. The work was supported by Grant AI 07007 from the United States Public Health Service, Bethesda, Maryland 20014.

† Dr. Unanue is supported by a Dernham Senior Fellowship of the American Cancer Society, California Division.

§ Dr. Cerottini was supported by United States Public Health Service Training Grant 5T1GM683. His present address is: Swiss Institute for Experimental Cancer Research, 21 rue du Bugnon, 1005 Lausanne, Switzerland.

plasma membrane of macrophages and includes experiments on its identification, physical properties, and, most important, of its immunogenicity. It will be shown that a few molecules of undegraded hemocyanin are retained on the membrane for long periods and escape catabolism. These molecules can react with extracellular antibody and can initiate an immune response. A great part of the immunogenic function of antigen-containing macrophages need not necessarily involve a processing step, but may be explained on the basis that these cells retain a small amount of relatively unaltered antigen accessible on their surface for long periods.

Materials and Methods

Mice.—A/J and CBA/J inbred mice (Jackson Laboratories, Bar Harbor, Maine) of 4–6 mos. of age and of either sex were employed.

Antigen.—The antigen was the hemocyanin from *Megathura crenulata*, keyhole limpets, (KLH)¹ purified by ultracentrifugation (78,000 *g* for 2½ hr in a Spinco Model L preparative ultracentrifuge) (13). In all experiments, the KLH fed to macrophages was in the associated form sedimenting at approximately 98S (14). Antibody determinations were made with dissociated KLH (14).

Iodination.—KLH or immunoglobulins were iodinated (¹³¹I or ¹²⁵I) by a chloramine T procedure (15) to a specific activity of 0.4–1.0 $\mu\text{Ci}/\mu\text{g}$.

Macrophages.—The macrophages were obtained from the peritoneal cavity of normal mice or mice 3 days after injection with peptone (1.5 ml intraperitoneally [i.p.] of 10% proteose peptone, Difco Laboratories, Inc., Detroit, Mich., Cat. No. 0121-01) (16). The handling and immunogenicity of KLH by equal number of macrophages from normal mice and mice injected with peptone were similar. The exudates from peptone-injected CBA mice contained around 10⁷ cells of which 80–90% were typical macrophages (16), while those from normal mice contained 3–5 $\times 10^6$ cells, of which half were macrophages.

Uptake of KLH.—The conditions of uptake varied somewhat and are explained in each experimental protocol. In general, two procedures were used: (a) *in vivo*, where the donors of macrophages were injected with 200 μg of radioactive KLH i.p. in 0.5 ml of Eagle's media (Grand Island Biological Co., Berkeley, Calif., Cat. No. 109G); the peritoneal exudates were harvested 20–30 min later; and (b) *in vitro*, where the macrophages (20–40 $\times 10^6$) and radioactive KLH (variable amounts from 20–1500 μg) were mixed in siliconized culture bottles in 6–8 ml of Eagle's media, usually with 5% fresh normal mouse sera. The mixture was incubated in suspension by stirring with a small magnet for 30–60 min at 37°C.

After uptake, the cells were washed three times and their content of radioactivity was determined in a gamma ray spectrometer.

Culture of Macrophages.—In most experiments, after the period of uptake of KLH the cells (5–10 $\times 10^6$) were cultured in plastic dishes (35 x 10 mm style, no. 3001, Falcon Plastics, Los Angeles, Calif.) with 1 ml of Eagle's media containing 5–10% normal mouse or fetal calf sera (Grand Island Biological Co. or Flow Laboratories, Inglewood, Calif.).

Immunogenicity.—The macrophage transfer systems previously described (4, 5, 16) was used with minor modifications. Live macrophages after uptake of KLH were injected i.p.

¹ *Abbreviations used in this paper:* ABC, antigen-binding capacity test; ABC-33, antigen-binding capacity test with 33% binding endpoint; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid tetra sodium; KLH, keyhole limpet hemocyanin; i.p., intraperitoneally; i.v., intravenously.

into syngeneic recipients. Usually, 14 to 27 days later, the recipients were bled, challenged with 50 μg of free KLH i.p., and bled again 7 days later. Antibody determinations were made on the sera obtained at the two bleedings.

Antibody Assay.—Antibodies to KLH were detected by an antigen-binding capacity test (ABC) using a coprecipitation method with anti-mouse globulin sera: 20 μl of test serum (or five-fold dilutions of it) were mixed with 20 μl containing 0.04 μg N of dissociated ^{125}I KLH for 16 hr in the cold; the mixtures were reacted with 200 μl of rabbit anti-mouse globulin sera (15 min at 37°C and 60 min at 4°C). The precipitates were washed three times with cold buffer and their radioactivity was determined. Calculations were made as indicated by Farr using the 33% binding end point (ABC-33) (17). The values are expressed as μg of KLH N bound per 1 ml of undiluted serum.

Antisera.—All antisera were produced in rabbits. A pool of anti-KLH sera was obtained from rabbits repeatedly immunized to KLH in Freund's adjuvant. Approximately 50% of the immunoglobulin G fraction of this antisera coprecipitated with an anti-KLH-KLH complex (18). Hyperimmune sera to bovine serum albumin (BSA) was similarly obtained. BSA was obtained from commercial source (Pentex Lab., Kankakee, Ill.). Anti-mouse globulins were obtained after 4–8 courses of immunization with mouse globulin in complete Freund's adjuvant.

The fractionation procedures to obtain the IgG fraction and the IgG fragments have been reported before (18).

Miscellaneous Reagents.—Trypsin, pancreatic ribonuclease A and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp., Freehold, N. J. Neuraminidase was obtained from Mann Research Laboratories, New York. Ethylenediaminetetraacetic acid tetra sodium (EDTA) was obtained from Pierce Chemical Co., Rockford, Ill.

Sucrose Density Gradients.—Radioactive materials were placed in gradients of 10–40% sucrose in saline. Gradients were run for 2½ and 15 hr in a Spinco Model L ultracentrifuge at 99,970 g (35,000 rpm) in an SW39 swinging bucket rotor. Fractions containing 15 drops of material were collected after puncturing the bottom of the tube.

RESULTS

Identification of KLH on the Surface of Macrophages.—The presence of antigen on the plasma membrane of macrophages in culture after the period of rapid catabolism was demonstrated by reacting a known anti-KLH antibody with macrophages in conditions where both the cytophilic properties of IgG and pinocytosis were minimized as reported in a preliminary communication (12).

30×10^6 macrophages were incubated in tissue culture bottles for 1 hr with 1 mg of KLH labeled with ^{125}I in media containing 5% normal mouse sera. The cells were then washed well and, in most experiments, planted in plastic dishes (usually 10^7 per dish) for 4–72 hr (in media devoid of KLH) before reacting with the labeled antibodies; thus, at the time of reaction with the antibody preparation, the cells had become attached to the dishes and had catabolized and eliminated around 80–90% of their content of KLH. In order to avoid the nonspecific interaction of intact IgG with macrophages, antibody monovalent and bivalent fragments were employed. Also, to insure that the reaction was taking place at the surface membranes, most of the experiments were carried out at 2–4°C. At this temperature pinocytosis is known to be inhibited (19). In each experiment ^{125}I -labeled fragments of rabbit anti-KLH IgG were mixed with 100- to 200-fold excess of either unlabeled anti-BSA IgG or, as control, unlabeled anti-KLH IgG. Before exposure to antibody, the cells were washed three times with media and then

mixed with 1 ml of the antibody preparations and incubated with gentle shaking, usually for 1 hr at 2–4°C. After incubation, the cells were again washed three times and detached by a rubber policeman and their content of ^{131}I and ^{125}I were determined. Table I and Figs. 1 and 2 detail representative experiments.

TABLE I
Binding of Anti-KLH F(ab')₂ to Macrophages

Antibody preparations			Number of cells per dish ($\times 10^6$)	Content ^{125}I -KLH μg	Fixation of antibody %
Anti-KLH F(ab') ₂ μg	Anti-BSA IgG μg	Anti-KLH IgG μg			
2.4	240	—	10	1.65	2.12
2.4	—	240	10	1.65	0.43
2.4	240	—	1	0.17	0.25
2.4	—	240	1	0.17	0.18

Macrophages (30×10^6) were exposed to 1 mg of ^{125}I -KLH in culture bottles for 1 hr, after which they were planted in dishes for 4 hr. The antibody preparations consisted of ^{131}I -anti KLH F(ab')₂ fragments mixed with either anti-BSA or anti-KLH IgG. In this experiment, the dishes contained 10^7 and 10^6 cells. The ratio of specific to nonspecific fixation was 5.1 and 1.4 with 10^7 and 10^6 cells, respectively.

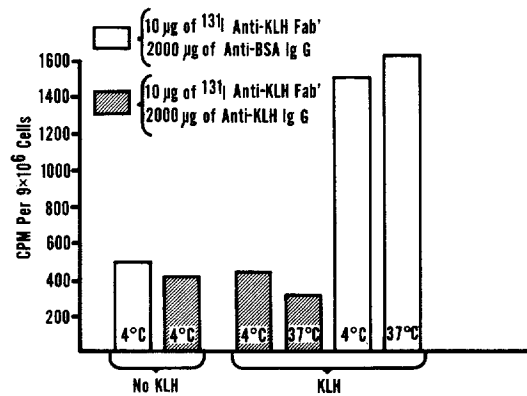


FIG. 1. Normal macrophages (No KLH) and macrophages that had been exposed to ^{125}I -labeled KLH (KLH) were cultured for 24 hr in antigen-free media in dishes. After washing, the cells were reacted with the ^{131}I -labeled anti-KLH Fab' mixed with an excess of either anti-KLH or anti-BSA IgG. In this experiment, the antibodies were incubated at either 4°C or 37°C. Cell associated radioactivity was determined after 1 hr incubation. Each bar is the mean of two cell cultures and represents the amount of antibody bound to macrophages (9×10^6) in each dish.

There appeared to be antigenic material on the macrophages that reacted specifically with Fab' or F(ab')₂ fragments of anti-KLH IgG. Note in Fig. 1 that the amount of antibody reacting nonspecifically was the same with macrophages exposed to KLH (a day before) as with normal, untreated macrophages.

The optimal time of incubation for demonstration of specific binding of antibody to the KLH-containing macrophages was 1 hr because at this time the ratios of specific to nonspecific fixation were highest (i.e., in Fig. 2 the ratios were 2.0, 4.4, and 2.9 after 5 min, 1 hr, and 5 hr of incubation, respectively). The total amount of antibody that could react with the cell-bound KLH was not determined. By reacting macrophages with larger amounts of antibody, there was an increase in total fixation, but also a disproportionate amount of non-specific uptake. Thus, a state of saturation was never reached. The highest

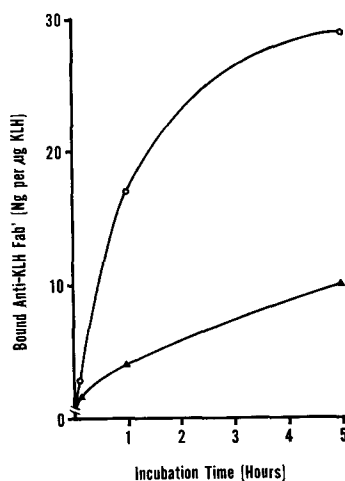


FIG. 2. Influence of time upon binding of antibody to antigen-containing macrophages. KLH-containing macrophages were cultured for 24 hr in antigen-free media and reacted for 5 min, 1 hr, or 5 hr with 1 ml of an antibody preparation containing 10 μg of ^{125}I anti-KLH Fab' mixed with either 2000 μg of unlabeled anti-KLH IgG (\blacktriangle — \blacktriangle) or 2000 μg of unlabeled anti-BSA IgG (\bullet — \bullet).

molar ratio of antibody-antigen that was obtained (assuming that KLH was associated and that the material reacting with antibody was one-fourth-one-fifth of the total present in the cell, see below) was around 20. The molar ratio of anti-KLH IgG to KLH at equivalence is 48 (14).

Most of the antibody that reacted with the macrophages (approximately 75%) was removable by treating the cells for 5 min with trypsin (0.2 mg in 1 ml). The amount of antibody reacting with the macrophage was the same on cells cultured from 4 to 72 hr after uptake of KLH, regardless of whether the test was carried out in cells cultured either in dishes or in suspension.

Removal of Retained KLH.—Attempts were made to remove the antigen that remained after the period of rapid catabolism with the use of several enzymes, different salt solutions, or chelating agents such as EDTA.

Macrophages were exposed to ^{131}I -KLH and then cultured for 24–72 hr in dishes in media free of KLH and containing 5–10% normal mouse sera or fetal calf sera. The cells were washed twice and reacted with one of the following: trypsin, 0.1 to 0.5 mg per ml of media for 5–15 min; pancreatic ribonuclease, 0.1 mg per ml for 15 min; neuraminidase, 10 $\mu\text{g}/\text{ml}$ for 15 min; EDTA, 0.001–0.1 M for 15–30 min. Some experiments also included treatment with 1 M NaCl or 0.1 M acetic acid for 5 min.

Radioactive material could be removed from live macrophages only by treatment with trypsin or with EDTA (Fig. 3). Around two-thirds of the removed radioactive material precipitated in 10% trichloroacetic acid. EDTA

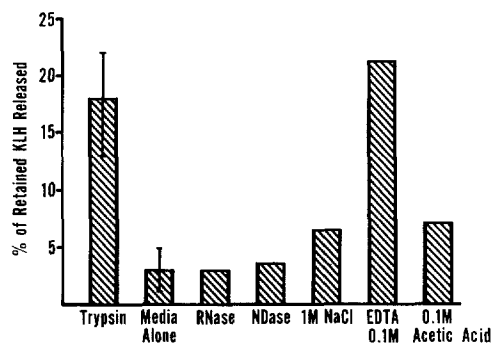


FIG. 3. The effect of various treatments on the antigen retained by macrophages. Macrophages that had been exposed to KLH were cultured for 24–72 hr prior to treatment. Media alone always released a small amount of radioactive antigen (around $\frac{1}{5}$ of that released by trypsin). RNase, ribonuclease, NDase, neuraminidase.

was most efficient at a concentration of 0.1 M. At 0.01 M, a concentration that should be effective in chelating all the Ca and Mg of the media, there was usually removal of 40–50% of the KLH removed with 0.1 M EDTA (the mechanisms by which EDTA removes KLH are not clear; macrophages can bind KLH when incubated in media devoid of Ca and Mg ions). The amount of material removed by trypsin was comparable in cells cultured from 4 hr to 7 days. There was no change in the viability of cells after treating with trypsin, EDTA, neuraminidase, and ribonuclease. 1 M NaCl and 0.1 M acetic acid affected markedly the viability of the cell.

Antigenicity of KLH Removed from the Live Macrophages.—Experiments were done to determine whether or not the material that was removed by treatment with trypsin was antigenic, i.e., would react with anti-KLH antibodies.

One experimental approach that was used was to determine if the radioactive material removable by trypsin would coprecipitate in an immune complex of KLH–anti-KLH antibodies. Macrophages that had been in culture in plastic dishes for 24 hr after uptake of ^{131}I -KLH were treated with trypsin (0.2 mg per ml per dish containing 10^7 cells for 5 min). The

supernatant to which was added 0.2 mg of soybean trypsin inhibitor was dialyzed for 18 hr against 0.15 M phosphate-buffered saline. The coprecipitation mixtures consisted of 400 μ g of rabbit anti-KLH IgG and 200 μ g of KLH plus 0.5 ml of the treated supernatant (around 0.5 μ g); a control precipitate consisted of a known anti-mouse globulin-mouse serum mixture.

A second experimental approach was to determine whether live macrophages that had been exposed to KLH could, after treatment with trypsin, still react with labeled anti-KLH antibodies. Macrophages that had taken up KLH were cultured for 24 hr in dishes, trypsinized, and were then exposed to the antibody mixtures (10 μ g of 125 I anti-KLH Fab' mixed with 2 mg of anti-BSA IgG and, as control, the same amount of labeled antibody mixed with 2 mg of anti-KLH IgG).

73% of the material that was removable by trypsin coprecipitated with an anti-KLH-KLH immune complex, while 11% precipitated in an unrelated immune complex. Also, live macrophages that were treated with trypsin reacted to a smaller extent or no longer with labeled anti-KLH antibodies. The extent of reaction of labeled antibody depended in part on the extent of trypsinization of the cells. Labeled antibody would no longer bind to macrophages treated with trypsin for 15 min, but would still bind to macrophages treated for 5 min (up to 60% less when compared to the amount bound to untreated macrophages exposed to KLH).

Size of the KLH Removed from the Macrophages.—The molecular size of the membrane-bound antigen was studied using the material obtained after treatment with trypsin or with 0.1 M EDTA.

Macrophages that had been in culture dishes for 24 hr after exposure to 125 I-KLH were exposed to trypsin (0.2 mg in 1 ml for 5 min) or to 0.1 M EDTA (for 20 min). To the material removed by trypsin was added 0.2 mg of soybean trypsin inhibitor. The supernatants were dialyzed for several hours against phosphate-buffered saline. Two tests were carried out on the supernatants and on control preparations of associated and dissociated KLH. In one, the materials were mixed with 5 mg of unlabeled associated KLH in 10 ml of saline and spun at 78,000 *g* for 2½ hr in a Spinco Model L ultracentrifuge. The amount of radioactive material in the upper 9 ml of the tubes was compared with the amount present in the precipitate and lower 1 ml. The second test was a sucrose density gradient analysis.

Most of the KLH in the supernatant from treated macrophages was of large molecular size, though heterogeneous. A significant part of the KLH was present in its associated form. In one experiment, for example, 91% of labeled, associated KLH precipitated when spun at 78,000 *g* for 3 hr as compared to 65% of the KLH from macrophages. In other experiments, density gradient analysis showed that approximately one-third of the KLH from macrophages sedimented as associated KLH, while the rest was of lower molecular weight and sedimented as the dissociated KLH (approximately 0.85×10^6 molecular weight) (Fig. 4). Similar results were obtained on the KLH released from macrophages incubated at 4°C for 1 hr (see next experiments). Thus, some of the KLH molecules appeared to dissociate upon interaction with the surface membrane of the cell.

Dose Relationship.—Attempts were made to determine the relationship between the dose of KLH fed to macrophages, the amount taken up, and the amounts retained after 24 hr.

A constant number of macrophages (35×10^6) were exposed to from 33 to 1506 μg of ^{131}I -KLH for 1 hr at 37°C (and in some experiments at 4°C), after which they were washed three

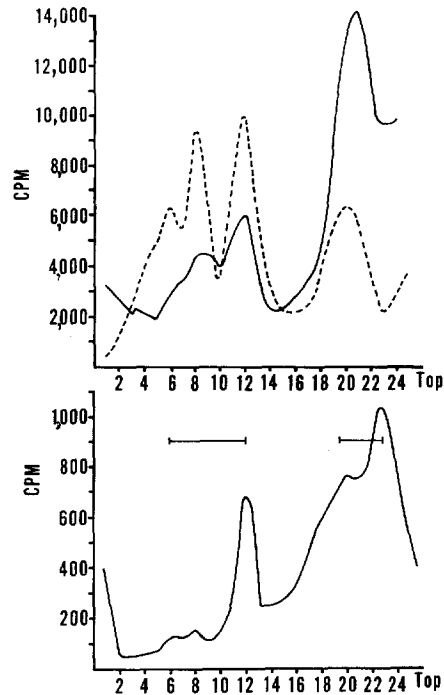


FIG. 4. Sucrose density gradients for $2\frac{1}{2}$ hr of the material released from macrophages after trypsin (upper graph) and 0.1 M EDTA (lower graph). Around $\frac{1}{3}$ of the material has a size comparable to associated KLH. The rest has a size comparable to dissociated KLH and sediments like it when gradients are run for 15 hr. The dotted curve in the upper graph represents free KLH- ^{131}I treated with trypsin. The bars in the lower graph represent the areas in the gradient of associated and dissociated KLH.

times, their radioactive content determined, and they were cultured in dishes (in media with 5% normal mouse sera and no KLH) for 24 hr. After 24 hr, the supernatant was collected, the cells were washed three times, and subjected to trypsin treatment. The amount of radioactivity in supernatant, in the trypsinized material, and in the cells was determined.

The results summarized in Table II indicate some reduction of the percentage of KLH taken up by the macrophages as the dose of antigen is increased. At the doses used, there was no preferential reduction in the amount of antigen

retained on the surface (i.e., removed by trypsin) or retained "inside" the cell at 24 hr.

Incubation of macrophages for 1 hr at 4°C led to uptake of around one-fourth of KLH taken up at 37°C. Most of the KLH (around 80%) was not pinocytosed, insofar as it could be effectively removed by treatment with trypsin. After uptake at 4°C and then culture at 37°C for 2–3 days, the amounts of KLH catabolized and retained by the cells were identical as in the above experiments where the uptake was first carried out at 37°C.

It was of interest to determine whether the fate of KLH in macrophages would be different if fed as part of an immune complex. An experiment similar to the one above was made by incubating the cells with 10 μg of KLH in pres-

TABLE II
Handling of Different Amounts of KLH by Macrophages

KLH $\mu\text{g}:35 \times 10^6 \text{ cells}$	Uptake (1 hr)		Retained in cells at 24 hr		Cell-bound KLH removed by trypsin
	%	μg	%	%	%
33	2.28	0.75	11.1	20.7	
126	1.88	2.36	10.5	15.9	
486	1.46	7.08	11.6	21.7	
1506	1.07	16.11	13.3	21.0	

Macrophages were exposed to the different amounts of KLH for 1 hr and cultured in media free of antigen for 24 hr. Per cent retained refers to the amount of KLH taken up that remained cell-bound 24 hr later. Per cent removed refers to the percentage of the cell-bound KLH at 24 hr that could be removed by trypsin. Each figure is the mean of two cultures. The percentage removed by trypsin has been corrected for the small amount removed by media alone (approximately 2–3% in this experiment).

ence of an amount of anti-KLH that would produce an insoluble complex. This led to approximately 8–10 times more uptake of KLH. The distribution in the macrophage of the KLH taken up as part of an immune complex was quite different insofar as only 2% of the material retained by the cell at 24 hr was removable by trypsin.

Immunogenicity of Membrane-Bound KLH.—The immunogenicity of the surface bound molecules of KLH was tested in the macrophage transfer system (4, 5, 16). Two experimental approaches were used: in one, the macrophages after uptake of KLH were treated in vitro at 2–4°C with antibody, after which they were washed well and transferred into syngeneic hosts. It was assumed that if antibody under these circumstances could "cover" enough of the antigen molecules bound at the surface it could abrogate the immune response to the macrophage-bound KLH. A second approach consisted of removing the surface-bound KLH by trypsin, after which the cells were transferred into syngeneic hosts. The following are representative experiments.

Peptone-injected CBA mice were injected i.p. with 200 μg of ^{131}I -KLH. Half an hour later the peritoneal macrophages were harvested, washed three times, and their radioactive content determined. Macrophages were incubated with gentle shaking at 4°C with either anti-KLH IgG or anti-BSA IgG. Each incubation mixture consisted of 100×10^6 macrophages containing 54 μg KLH with 2 mg of antibody preparation in 5.5 ml. After 1 hr, the cells were washed

TABLE III
Effect of Treating Macrophages with Antibody or Trypsin Prior to Transfer

Macrophages			Recipients			Mean antibody titer
Number ($\times 10^{-6}$)	Amount of KLH	Treatment	Strain	Number	Day of secondary challenge	
	μg					
7.3	4.0	anti-BSA	CBA	8	20	11.3
7.3	4.0	anti-KLH	CBA	8	20	1.8
0.7	0.4	anti-BSA	CBA	8	20	2.4
0.7	0.4	anti-KLH	CBA	8	20	<0.6
3.5	2.0	anti-BSA	A	6	7	14.9
3.5	2.0	anti-KLH	A	6	7	8.2
3.5	2.0	anti-BSA	A	6	20	20.8
3.5	2.0	anti-KLH	A	6	20	11.5
5.6	1.2	—	A	6	27	9.5
6.0	0.7	trypsin	A	6	27	2.4
2.1	2.1	—	CBA	6	20	3.2
4.7	2.1	trypsin	CBA	6	20	1.2

Anti-BSA: rabbit anti-bovine albumin IgG; Anti-KLH: rabbit anti-KLH IgG.

Experiments are detailed in text. Difference between each experimental and control group was statistically significant ($P < 0.05$ – 0.01). Antibody titers are expressed as μg of KLH N bound per ml of undiluted serum (ABC-33). The antibody titers were from sera obtained 7 days after the secondary challenge.

three times, counted again, and injected i.p. into syngeneic CBA hosts. 20 days later the recipients were boosted with 50 μg of KLH i.p. and bled for antibody 7 days later.

A second experiment utilized A mice, a strain that usually responds strongly to KLH. Uptake of KLH was performed *in vitro*, since the yields of macrophages and uptake of KLH *in vivo* are not as high as in CBA. (71×10^6 macrophages were incubated with 1 mg of KLH in total volume of 8 ml for 1 hr at 37°C.) The cells after washing were incubated with the antibody preparations (100×10^6 macrophages containing 58 μg of KLH with 2 mg of either anti-KLH IgG or anti-BSA IgG in 5.5 ml for 1 hr at 4°C). The cells after washing were injected i.p. into recipients that were boosted 7 or 20 days later with 50 μg of free KLH.

A control experiment consisted of incubating 35×10^6 normal macrophages with 500 μg of either antibody. As before, after 1 hr the cells were washed and injected together with 0.5 μg of free KLH i.p. The mice were challenged 20 days later with 50 μg of KLH and bled 7 days later for antibody determinations.

Some of the results are summarized in Table III. Incubation of the macrophages *in vitro* with anti-KLH markedly decreased their immunogenicity. This was most evident in the CBA mice which upon secondary challenge made less than 10% as much anti-KLH antibody if primed with macrophages incubated *in vitro* with anti-KLH IgG as with anti-BSA IgG. In the experiment with CBA mice, before the secondary challenge, the sera of mice receiving macrophages incubated with anti-BSA IgG had low amounts of antibody capable of binding 8.1% of KLH in the ABC test at a 1:2 dilution of serum. (The range was between 4.2 and 10.9%; these values are corrected for the degree of binding of normal mouse sera which was 0.7%.) The mice that received macrophages incubated with anti-KLH IgG, however, had no binding of KLH over the background control sera. Transfer of normal macrophages incubated *in vitro* with anti-KLH or anti-BSA IgG together with 0.5 μ g of KLH resulted in comparable secondary immune responses, indicating that the results mentioned above could not be explained by a mere transfer of cytophilic anti-KLH antibodies in the macrophages (ABC-33 titers of 49 and 40, respectively, which were not statistically significant).

Immunogenicity of macrophages treated with trypsin in order to remove a great part of the antigen bound to the plasma membrane was tested. Two representative experiments are detailed.

Macrophages from A mice were incubated *in vitro* with ^{131}I -KLH (30×10^6 cells, 1 mg of KLH in culture bottles) for 30 min and washed three times. One portion of cells was trypsinized (10 min with 0.2 mg of trypsin per ml). Six mice received 6×10^6 trypsinized cells containing 0.65 μ g of KLH and six received 5.6×10^6 normal cells containing 1.2 μ g KLH. All mice were challenged 27 days later with 10 μ g of free KLH and bled for antibody determinations 7 days later.

In a second experiment uptake of KLH by macrophages was done *in vivo*. CBA mice were injected with 200 μ g of ^{131}I -KLH *i.p.* and the peritoneal exudates harvested 20–30 min later. A sample of cells was treated with trypsin (0.2 mg per ml) for 30 min, after which it was washed twice with Eagle's medium containing 0.2 mg of soybean trypsin inhibitor and transferred *i.p.* into recipients. Recipients were boosted 20 days later with 50 μ g of KLH and bled for antibody 7 days later.

A control experiment included a study of the immunogenicity of macrophages trypsinized before exposure to KLH. 30×10^6 macrophages from A mice were trypsinized (10 min with 0.2 mg of trypsin per ml) and then incubated with ^{131}I -KLH. After 1 hr, the cells were washed, their radioactivity determined, and then they were transferred *i.p.* into syngeneic hosts.

As shown in Table III, two-thirds of the immune response was abrogated by trypsinization of the macrophages after uptake of KLH. In the second experiment, for example, we could assume that most of the antigen in the trypsinized cells was intracellular, while the normal cells contained around one-fourth–one-fifth of the antigen on the membrane. The presence of more antigen on the membrane at the time of transfer, therefore, resulted in a much higher antibody response. On the other hand, pretrypsinized macrophages containing KLH in several experiments induced responses comparable to those of non-

trypsinized cells containing KLH. Also, the capacity of trypsinized and normal macrophages to migrate to the spleen was studied with the use of phagocytosed ^{198}Au as a tracer. The amount of radioactive material in the spleen of mice injected intravenously (i.v.) or i.p. with normal and trypsinized macrophages containing ^{198}Au were comparable at 24 and 48 hr.

DISCUSSION

These and other recent experiments have delineated some of the pathways that soluble antigens like hemocyanin follow when in contact with the phagocytic cells of the reticuloendothelial system. After uptake, most of the hemocyanin was rapidly catabolized and breakdown products were eliminated from the cell. However, a small amount was retained with little change in amount for many days. It was shown previously by different experimental approaches that the immunogenicity of macrophage-bound hemocyanin was associated with the small persistent fraction of antigen (4). All indications are that a part of this retained antigen remains on the membrane of the cell. The evidence for this is several-fold: (a) antibody can specifically bind to the macrophages that have been exposed to the antigen hours or days before; the antibody can bind under conditions where there is no active process of uptake such as pinocytosis; (b) this antibody can be recovered after treatment of the macrophages with trypsin, a treatment which apparently is only affecting the surface membrane of the cell; (c) trypsin treatment removes radioactive antigen from the macrophages; (d) the antigen removed by trypsin is that to which the antibody binds; and finally, (e) radioautographic studies have disclosed hemocyanin associated with the surface of macrophages (12). The presence of large molecular weight antigen bound to the membrane of the cell for long periods is of obvious importance insofar as the surface membrane is accessible to potential antibody-forming cells. An explanation for the retention of a few molecules of antigen on the membrane for prolonged periods, however, is not available. So far, enhancement of pinocytosis with agents like heparin or dextran sulfate has not resulted in the engulfing of these few molecules. Whether the antigen is bound to peculiar areas of the membrane that do not participate in endocytosis remains a possibility.

Three points should be considered in assessing the significance of the *in vitro* persistence of hemocyanin on the macrophage surface. A first consideration should be whether or not this phenomenon also occurs *in vivo* or is merely the result of tissue culture conditions. We have observed (unpublished observations) that either peritoneal macrophages cultured *in vivo* in diffusion chambers implanted in the abdominal cavity, or macrophages from spleens of mice injected days previously also contain a small amount of antigen removed by trypsin. The failure of others to detect *in vivo* the few molecules of antigen on the surface of macrophages (20) may be due to sensitivity of techniques

and/or the nature of the antigens. The technique which has been used most frequently to trace antigen, radioautography, may not be sensitive enough to detect the 1–3 molecules on the surface of the macrophages per 100 taken up. Moreover, some antigens which aggregate *in vivo* are mostly taken up into the vesicles of the macrophages. In our experiments, for example, KLH, when complexed to antibody, was mostly endocytosed. Secondly, is the surface fixation of antigen peculiar to peritoneal macrophages? This does not seem to be the case, since dendritic cells of germinal centers, which apparently are different from macrophages, retain antigen (complexed with antibody) on their surface (reviewed in reference 1). The immunogenicity of antigen bound to dendritic cells, however, has been difficult to determine, since these cells cannot be isolated as the macrophages. A final consideration is whether the retention of antigen in the membrane is an exclusive peculiarity of the hemocyanins. Preliminary experiments have shown that other foreign soluble antigens like gamma globulins may also be retained on the membrane for prolonged periods. However, more studies need to be carried out with different antigens varying in their physicochemical properties in order to critically assess this point.

The experimental evidence suggests that the KLH residing on the plasma membrane of macrophages is immunogenic. If at the time of transfer of live macrophages containing KLH, the antigen present on the surface is either covered by antibody, or in great part removed by trypsin, there is marked reduction of immunogenicity, despite the fact that the bulk of KLH (or equivalent products) still remains in an intracellular site. The immunogenicity of the intracellular KLH cannot be determined because in no situation (including the experiment in which cells were trypsinized) was the cell-bound KLH exclusively either on the surface or intracellular. This is an important consideration in view of the many reports which show that the material obtained from vesicles or extracted from macrophages is immunogenic. Whether these intracellular products can become accessible to the antigen-sensitive cells *in vivo* remains to be determined.

The KLH bound to the plasma membrane was of large molecular weight, *i.e.*, in its associated and dissociated forms. It was previously found as a result of experiments involving two trypsin treatments of macrophages that most, if not all, the membrane-bound KLH was derived from native, free KLH and did not result from antigen previously taken into vesicles (12). If the surface-bound molecules of KLH at the time of interaction with an antigen sensitive cell have the same characteristics as determined *in vitro*, which seems likely, it means that the immunogenic moiety is far from being a processed fragment. It is apparent that the plasma membrane is a site where some molecules of antigen escape degradation and can, therefore, retain their original structure. Hence, the antibody response to macrophage-bound antigen may still be directed to conformational determinants of the native antigen.

By virtue of its effect on the antigen, the macrophage can be considered the first regulator of the immune response, since it may determine the size of the immunogenic stimulus. If we accept that the immunogen is derived from some of the antigen retained by the macrophage, it means that the major portion (>80–90%) of the antigen taken by the reticulendothelial system is catabolized and lost to the antigen-sensitive cell. Only when future experiments outline all possible pathways of interaction of antigen with the responding lymphocytes will the importance of the dual role of macrophage in destroying and retaining immunogen be better evaluated. The observations that macrophages at the appropriate lymphoid sites may concentrate and conserve a small amount of an immunogen like hemocyanin, in a form probably close to its native state and potentially accessible to immunological recognition, might largely explain their role in the induction and maintenance of an immune state.

SUMMARY

Macrophages were cultured for several hours after a brief exposure to radioiodinated keyhole limpet hemocyanin. Most of the hemocyanin taken up by the macrophages was rapidly catabolized and eliminated from the cell. A few molecules were retained on the plasma membrane of the cells for prolonged periods and were not subject to endocytosis and catabolism. These few molecules of hemocyanin bound to the plasma membrane were identified by observing the fixation of antibody fragments to macrophages at low temperature. The membrane-bound antigen, which could be removed by trypsin or EDTA, was of large molecular size, though heterogeneous. A great part of the immune responses of mice to hemocyanin bound to live macrophages could be abrogated by treatment of the macrophages in vitro with antibody or trypsin. Hence, most of the immunogenicity of hemocyanin bound to macrophages was attributed to the few molecules of antigen bound to the plasma membrane.

We appreciate the advice of Dr. F. J. Dixon throughout this work. We thank Mrs. P. McCaffery, Miss K. Lorenz, and Miss R. Coss for skillful technical assistance.

BIBLIOGRAPHY

1. Humphrey, J. H. 1969. The fate of antigen and its relationship to the immune response. The complexity of antigens. *Antibiot. Chemother.* **15**:7.
2. Gallily, R., and M. Feldman. 1967. The role of macrophages in the induction of antibody in x-irradiated animals. *Immunology.* **12**:197.
3. Mitchison, N. A. 1969. The immunogenic capacity of antigen taken up by peritoneal exudate cells. *Immunology.* **16**:1.
4. Unanue, E. R., and B. A. Askonas. 1968. Persistence of immunogenicity of antigen after uptake by macrophages. *J. Exp. Med.* **127**:915.
5. Unanue, E. R. 1969. The immune response of mice to keyhole limpet hemocyanin bound to macrophages. *J. Immunol.* **102**:893.
6. Fishman, M., and F. L. Adler. 1963. Antibody formation in vitro. *In* Third Inter-

- national Symposium on Immunopathology. P. Grabar and P. Miescher, editors. Schwabe & Co., Basel. 79.
7. Askonas, B. A., and J. M. Rhodes. 1965. Immunogenicity of antigen containing ribonucleic acid preparations from macrophages. *Nature (London)*. **205**:470.
 8. Friedman, H. P., A. B. Stavitsky, and J. M. Solomon. 1965. Induction in vitro of antibodies to Phage T2: antigens in the RNA extract employed. *Science (Washington)*. **149**:1106.
 9. Gottlieb, A. A., and P. Doty. 1967. Studies on macrophage RNA involved in antibody production. *Proc. Nat. Acad. Sci. U.S.A.* **57**:849.
 10. Roelants, G. E., and J. W. Goodman. 1968. Immunochemical studies on the poly- γ -D-glutamyl capsule of *Bacillus anthracis*. IV. The association with peritoneal exudate cells ribonucleic acid of the polypeptide in immunogenic and nonimmunogenic forms. *Biochemistry*. **7**: 1432.
 11. Roelants, G. E., and J. W. Goodman. 1969. The chemical nature of macrophage RNA-antigen complexes and their relevance to immune induction. *J. Expt. Med.* **130**:557.
 12. Unanue, E. R., J.-C. Cerottini, and M. Bedford. 1969. The persistence of antigen on the surface of macrophages. *Nature (London)*. **222**:1193.
 13. Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Sossdorf. 1963. Methods in Immunology. W. A. Benjamin, Inc., New York.
 14. Weigle, W. O. 1964. Immunochemical properties of hemocyanin. *Immunochemistry*. **1**:295.
 15. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunological studies. *Int. Arch. Allergy Appl. Immunol.* **29**:189.
 16. Unanue, E. R., and B. A. Askonas. 1968. The immune response of mice to antigen in macrophages. *Immunology*. **15**:287.
 17. Farr, R. S. 1958. A quantitative immunochemical measure of the primary interaction between I^{*}-BSA and antibody. *J. Infec. Dis.* **103**:239.
 18. Cerottini, J.-C., P. J. McConahey, and F. J. Dixon. 1969. Specificity of the immunosuppression caused by passive administration of antibody. *J. Immunol.* **103**:268.
 19. Cohn, Z. A. 1968. The structure and function of monocytes and macrophages. *Advan. Immunol.* **9**:163.
 20. Nossal, G. J. V., A. Abbot, and J. Mitchell. 1968. Antigens in immunity. XIV. Electron microscopic radioautographic studies of antigen capture in the lymph node medulla. *J. Exp. Med.* **127**:263.