PERSISTENCE OF IMMUNOGENICITY OF ANTIGEN AFTER UPTAKE BY MACROPHAGES

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Macrophages have the important function of participating with other cells like polymorphonuclear leukocytes (PMNs) in the uptake of bacteria, virus, and miscellaneous foreign proteins. After the uptake of these materials, both macrophages (1, 2) and PMNs (3) can catabolize them in a rapid and efficient way. (There are important exceptions however; macrophages do not catabolize well *Mycobacterium tuberculosis* [4], pneumococcal polysaccharide [5], or synthetic polymers of D-amino acids [6]). In addition, live macrophages, unlike PMNs, can interact with and induce syngeneic lymphoid cells to synthesize specific antibody (7–10). Cohn made an early attempt to correlate both functions, i.e. catabolic and immunogenic, comparing PMNs and macrophages after uptake of ^{32}P-labeled *Escherichia coli* (11). He noted that the catabolism of most of the bacteria proceeded at similar rates in both cell types for a 2 hr period of observation, but that macrophages, in contrast to PMNs, showed no decrease in immunogenicity. Previously, other investigations had indicated that immunogenic material could be extracted from macrophages days or weeks after uptake of protein antigens (2, 12–15); the information, however, was mostly qualitative and its relevance remained uncertain.

In the present study, we have attempted to correlate the uptake, catabolism, persistence, and immunogenicity of antigen in macrophages. Hemocyanin from *Maia squinado* (MSH) labeled with ^{131}I was used as antigen. The macrophages were obtained from peritoneal exudates after the injection of proteose peptone and after in vivo uptake of MSH. Immunogenicity was tested by studying the immune response of syngeneic mice after transfer of live macrophages containing antigen. In X-irradiated recipients such macrophages induced synthesis of antibody only if lymphoid cells from normal donors were also transferred (10); thus, induction of antibody synthesis was dependent upon the interaction of macrophages and lymphoid cells.

The experiments to be described show that the immunogenicity of live macrophages containing antigen persists for prolonged periods of time and is asso-

ciated with a small percentage of antigen held in a form protected from rapid breakdown and elimination.

Materials and Methods

Experimental Animals.—Female CBA mice (20–25 g) kept as an inbred line at the National Institute for Medical Research were used in all experiments.

Antigen.—The antigen was the hemocyanin from *Maia squinado* (MSH) which on analytical ultracentrifugation at pH 7.4 contained a main sedimenting component of 26S and a small amount of heavier and lighter material (2).

Isotope Labeling.—MSH was labeled with ^{131}I or ^{125}I (from Radiochemical Centre, Amersham, England) by a chloramine-T procedure (16) to a specific activity of 0.5–1.0 $\mu c/\mu g$.

Antibody Determinations.—Antibodies to MSH were determined by hemagglutination using bis-diazotized benzidine (17) to couple MSH to sheep red blood cells (18). Each serum sample was serially diluted (in Veronal buffer containing 1% rabbit serum) in 2-fold steps starting at an initial dilution of either 8-, 20-, 40-, or 50-fold. Controls consisted of normal mouse serum and a serum containing a known amount of antibody to MSH. In all experiments, sera from individual mice were titrated. Results were expressed as the geometric mean of the reciprocal of the highest dilution of serum which gave a positive hemagglutination reaction.

In some experiments, the antibodies were titrated after reduction and alkylation. The sera from a given experimental group were pooled and treated for 2 hr at room temperature with 0.1 M mercaptoethanol followed by 0.1 M iodoacetamide. Previous studies in this strain of mice have indicated a good correlation between the mercaptoethanol sensitivity of the antibodies and their molecular size as determined by sucrose gradient ultracentrifugation (10).

Macrophages.—Peritoneal cells were harvested from the peritoneal cavity of 25 g mice 3 days after an intraperitoneal injection of 1.5 ml of 10% proteose peptone. There was no attempt to purify the population of cells which consisted of 80–90% macrophages (large phagocytic cells with pale, sometimes reniform, nuclei and cytoplasm containing variable inclusion bodies) (1, 10). The remaining cells were lymphocytes of variable sizes and mast cells. Previous transfer studies have shown that the population of peritoneal macrophages induced with peptone broth is not contaminated with antibody-forming cells in sufficient number to induce, by itself, an immune response in irradiated hosts (10).

PMNs.—A PMN-rich exudate was obtained from the peritoneal cavity of mice injected *3 hrs* previously with 1.0 ml of 10% proteose peptone intraperitoneally. The exudate was composed of approximately 60% PMNs and 15% macrophages, the remaining cells were lymphocytes and mast cells.

Uptake of ^{131}I-MSH by Peritoneal Cells.—100 μg of ^{131}I-MSH were injected intraperitoneally into appropriate mice (in most experiments to mice which 3 days previously had received proteose peptone intraperitoneally and consequently had a peritoneal exudate made mostly of macrophages). The peritoneal cells were harvested 30–45 min later, spun at 500 g for 10 min at 4°C, and then washed thrice with cold Eagle's culture medium. The radioactivity of the cells was determined in a NaI crystal scintillation counter. Generally 0.3–1.0% of the material injected intraperitoneally was taken up by macrophages representing 0.3–1.0 μg of ^{131}I-MSH/5–8 × 10^6 cells.

Lymph Node Cells.—Axillary, submaxillary, aortic, and inguinal lymph nodes were removed from normal mice. A cell suspension was made by gently pressing the nodes through a nylon sieve.

Tissue Culture of Cells.—Peritoneal cells were harvested after the in vivo uptake of ^{131}I-MSH and washed thrice with Eagle's medium. As the medium from the last wash did not contain an appreciable amount of radioactivity, it was assumed that all the antigen at the

start of the culture was firmly held by the cells. The antigen-containing cells (20×10^6) were maintained in siliconized screw top bottles (25 ml volume) containing 6 ml of Eagle's medium buffered with 0.1 M tris buffer [tris(hydroxymethyl)aminomethane] to pH 7.2 and 5% v/v normal mouse serum, and kept in suspension by stirring slowly with a magnetic stirrer. Under these conditions, cells did not adhere to the glass and for 48 hrs maintained well. After that time, progressive clumping and increase in granularity of cytoplasm resulted.

Radioautography.—For radioautography, Ilford L4 liquid nuclear emulsion was applied to cell smears. The slides were exposed for 5–15 days at which time they were developed using routine procedures.

Irradiation.—Mice were irradiated with 600 or 660 R of whole body irradiation (5.5 ma; dose rate of 37 R/min at 32 cm fom the target).

Method Used to Study the Immune Response to MSH.—MSH did not elicit a primary immune response in the doses employed, 0.10–1.0 μg, and without the use of adjuvants. The immunogenicity of MSH, either in soluble form or when held in macrophages, was therefore assayed by its capacity to "prime" a normal mouse for a secondary immune challenge (10). Macrophages containing a known amount of ^{131}I-MSH, or soluble MSH, were injected intraperitoneally into groups of 6–9 CBA mice; 3 wk later the mice were challenged with 1 μg of soluble MSH intraperitoneally (a dose which did not induce circulating antibody in unprimed mice); serum was collected 8 days later for estimation of antibody titers. The type and amount of antibody obtained after the secondary challenge was proportional to the amounts of MSH given in the priming injection (10).

EXPERIMENTAL RESULTS

Fate of MSH After Uptake by Macrophages.—

Peritoneal macrophages were harvested 45 min after intraperitoneal injection of ^{131}I-MSH, and maintained in tissue culture as described under Materials and Methods. After varying periods of time, the cells were separated from the incubation medium by centrifugation and washed thrice with tissue culture medium. The ^{131}I radioactivity present in the cells (20×10^6) and in the tissue culture fluid was estimated before and after precipitation in 10% trichloroacetic acid (TCA) to give an indication of the extent of degradation of the ^{131}I protein.

The macrophages rapidly catabolized a major part of the ^{131}I-MSH taken up. During the first 2–3 hr of tissue culture, 70–80% of the radioactivity of MSH was lost from the cell and appeared extracellularly—80–90% of the ^{131}I material was not precipitable with TCA and thus represented degraded protein. Around 10% of ^{131}I protein taken up was retained in the cells after 5 hr (of which 60–75% was precipitable by TCA) and only very little of this was lost during the next 2 days of tissue culture; it thus appeared to be held by the cell and protected against rapid catabolism (Table I).

Radioautographic studies were done in order to determine the proportion of macrophages which contained ^{125}I-MSH before and after several hours of culture. Cell smears, prepared immediately after in vivo uptake of ^{125}I-MSH and after 4 hr of tissue culture were set up for radioautography. Immediately after collection, about 75% of the peritoneal macrophages contained MSH, the radioautography grains being unevenly distributed over the cell and varying in number from cell to cell. After 4 hr of culture, 69% of the cells still contained grains, which were reduced in number. Of these, 25% had grains situated mainly

in the peripheral areas of the cytoplasm near the cell edge; 10% had grains localized only in the cytoplasm well separated from the peripheral areas and 65% had grains situated over inner cytoplasm and peripheral areas of the cell. The exact intracellular localization, however, was impossible by light microscopy. This experiment has been extended employing electron microscopy radioautography and will be reported subsequently.

For comparative purposes, the uptake of MSH was also studied in PMNs which are not known to participate in the immune response (11, 19). A PMN-rich exudate containing MSH was obtained from 3 hr peptone-stimulated mice injected intraperitoneally with 100 μg of MSH. The uptake of MSH was 0.32% of the dose injected which was similar to that obtained with macrophage rich

TABLE I

Fate of ^{131}I-MSH after Uptake by Macrophages

Tissue culture	Cells			Tissue Culture Fluid	
	Total MSH	Protein-bound	Cells containing MSH	Total MSH	Protein-bound
hr	%	%	%	%	%
0	100	60–70	75	0	—
2	20–30	50		70–80	14–20
4–5	9–14	60–75	69	86–91	10–14
21	9–14	60–75		86–91	10–14

This table represents the results of several experiments. Macrophages containing ^{131}I-MSH after in vivo uptake (= 100% of MSH) were cultured for the above periods of time. Percentage of protein-bound MSH was obtained after precipitation of the material in 10% TCA. The percentage of cells containing MSH was obtained by radioautography using ^{125}I-MSH.

exudates. The cells were set in tissue culture and studied as above but only in samples obtained 1–5 hr after culture. PMN-rich exudate steadily degraded the MSH to a level of 3% at 5 hr.

Duration of Immunogenicity of MSH after Uptake by Macrophages.—

In the previous section, it was noted that a large part of ^{131}I-MSH taken up by the macrophages was catabolized within 5 hr. We now attempted to correlate cellular antigen content with immunogenicity at various time intervals after antigen uptake, using three different experimental conditions: 1) Macrophages containing known amounts of ^{131}I-MSH were maintained in tissue culture for several hours, and then transferred into normal syngeneic mice in order to estimate their immune response. As the tissue culture conditions, however, were not favorable after 48 hr and as we wanted to assay for immunogenicity for longer periods of time after uptake of antigen, the following experiments were designed: 2) Macrophages containing ^{131}I-MSH harvested 30–45 min after in

vivo uptake were transferred to mice which had received 660 R of whole body irradiation the day before. Previous study had shown that such recipients would develop a very poor immune response but that, if injected shortly after with lymph node cells (5×10^6), this immune response would be reconstituted. In the present experiments, the irradiated mice were reconstituted by injection of normal lymph node cells several hours or days *after* transfer of the macrophages. It was assumed that if immunogenic material persisted in the
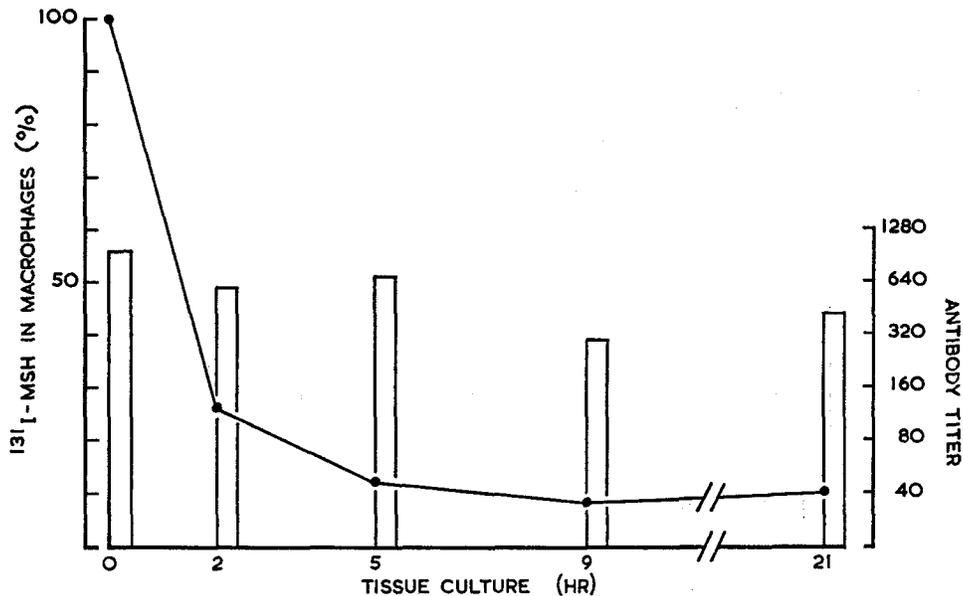

FIG. 1. Macrophages were obtained after in vivo uptake of ^{131}I-MSH and were set in tissue culture for the times shown on the abscissa. The curve represents the percentage of MSH in 20×10^6 cells. 100% is equivalent to the radioactivity in the cells just prior to culturing. The bars represent hemagglutination titer: 3×10^6 macrophages were transferred to syngeneic mice which were challenged 3 wk later with 1 μg of soluble MSH and bled for serum 8 days later.

transferred macrophages, the lymph node cells injected subsequently would be capable of interacting with it and would become primed; 3) macrophages containing MSH were transferred to mice, irradiated with a lower dose (600 R) which were then permitted to recover spontaneously from the irradiation injury. At variable times after transfer of the macrophages, the mice were given a secondary challenge and 1 wk later serum was collected to determine whether priming had taken place.

The detailed experimental protocol and results follow:

1. Macrophages harvested 30–45 min after in vivo injection of 100 μg ^{131}I-MSH were maintained in tissue culture as described under Materials and Methods. At varying intervals

(up to 21 hr) the cells were washed with tissue culture medium, and their radioactivity determined. A constant number of macrophages (3 × 10⁶ cells) before and after tissue culture were transferred to groups of 6–9 normal mice in order to assay their immunogenic properties (Fig. 1).
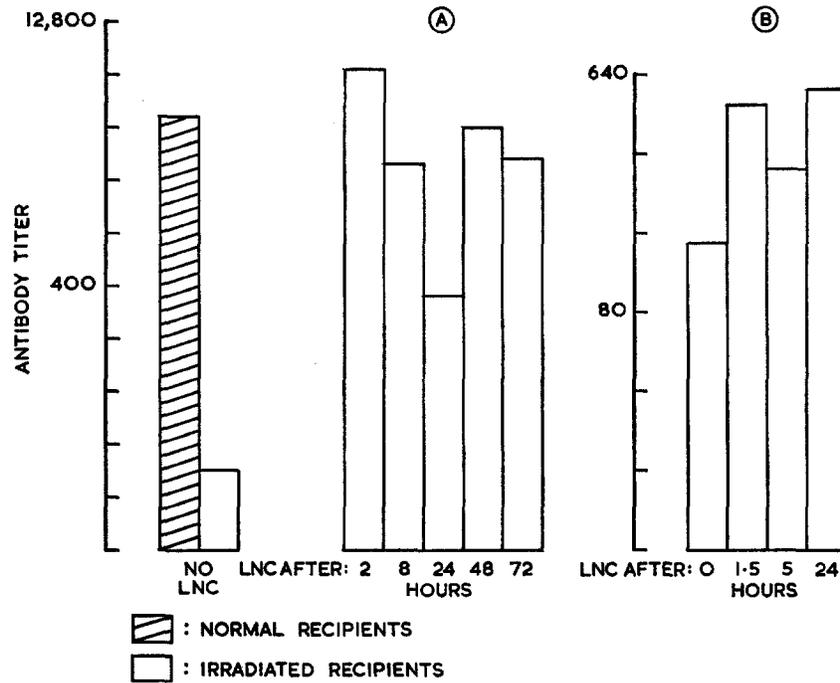

FIG. 2. Two experiments in which macrophages containing MSH were transferred to normal and irradiated mice. Some of the irradiated mice were then reconstituted with lymph node cells (LNC) at different times after the transfer of macrophages. All mice were challenged 3 wk later with 1 μg of soluble MSH. The hemagglutination titers were obtained 8 days after the secondary challenge.

3 × 10⁶ cells had taken up an equivalent of 0.3 μg of ¹³¹I-MSH. After tissue culture, for 2, 5, 9, and 21 hr, they contained ¹³¹I material equivalent to 0.09, 0.04, 0.03, and 0.04 μg of the original ¹³¹I-MSH respectively. This is an over-estimate, since only 60–75% of this intracellular ¹³¹I material is precipitable with TCA, and the rest appears to be free ¹³¹I. Despite this 10-fold difference in antigen content, there was no difference in antibody response elicited by the macrophages transferred at the various time intervals after uptake. Such a difference in the content of *immunogenic* material, however, would have been reflected in a significant decrease in antibody titer (10).

2. Two representative experiments are illustrated in Fig. 2. In one of the experiments (A), 2.2 × 10⁶ macrophages containing 0.3 μg of MSH (measured immediately after in vivo up-

take) were transferred to mice which 24 hr before had received 660 R of whole body irradiation. 2, 8, 24, 48, and 72 hr after the transfer of macrophages, the mice received 5×10^6 lymph node cells from normal mice intraperitoneally. In another experiment (B), 3.7×10^6 macrophages containing 0.5 µg of MSH were transferred to the irradiated mice which then received the lymph node cells simultaneously or 1.5, 5, and 24 hr later. Control groups consisted of normal mice and irradiated mice not reconstituted with lymph node cells into which macrophages containing MSH were transferred. All groups, consisting of 6–9 mice, were challenged 20 days after transfer of macrophages with 1 µg of soluble MSH and were bled for antibody determinations 8 days later.

The results indicated that lymph node cells were effectively primed when transferred as long as 72 hr after the macrophages. Although there was some
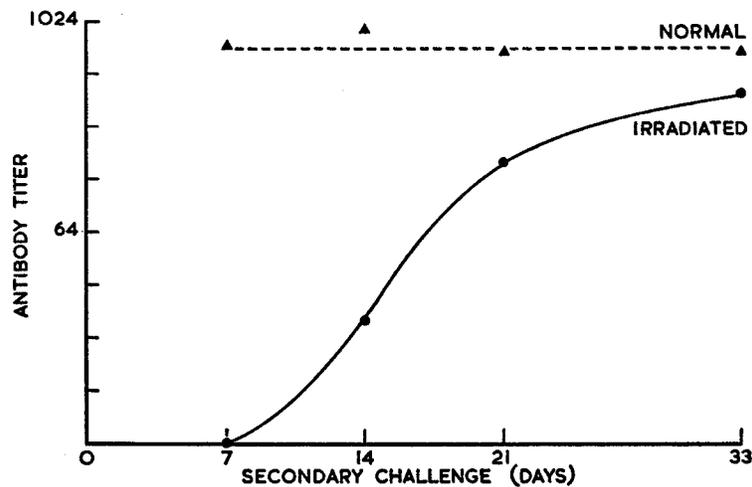

FIG. 3. Macrophages containing MSH were transferred into normal or irradiated mice which were challenged with soluble MSH at times shown on the abscissa. The serum antibody was determined 7 days after the challenge.

variability in antibody titers between groups, the pattern of response did not demonstrate any striking loss of immunogenicity by the MSH persisting in the transferred macrophages. The variability of antibody response from group to group probably reflected the fact that different batches of lymph node cells were prepared for each time point for transfer. The proportion of antibody sensitive to mercaptoethanol (19S) was similar in all groups (2–3-fold reduction in $\log_2$ titers).

3. Normal and irradiated mice received 2.1×10^6 macrophages containing 0.13 µg of MSH (measured immediately after in vivo uptake). The irradiated mice had received 600 R of whole body irradiation, a day prior to cell transfer. 7, 14, 21, and 33 days later, each group was challenged with 1 µg of soluble MSH and then bled for antibody determinations 7 days later.

The results, illustrated in Fig. 3, indicated that the irradiated mice made no immune response or a negligible one when challenged during the first 2 wk after transfer of antigen in macrophages. By 21 days, at a time when immunological recovery was expected (20), an immune response was clearly detected and this increased to near normal levels by 33 days. At day 21, all the antibodies were mercaptoethanol sensitive; at day 31, both mercaptoethanol-sensitive and -resistant antibodies were made. The latter was comparable to that observed in normal recipients when challenged 7–33 days after transfer of the macrophages.

Despite the known fact that the irradiated mice might have had an increased immune response during recovery (21, 22), the results still clearly indicated that immunogenic material must have persisted for at least 2 wk after the original transfer.

DISCUSSION

It has been shown in this study that immunogenicity of live macrophages persisted relatively unaltered for long periods of time, and appeared to be associated with only a small percentage of antigen held by the cell in a form where it was protected from rapid breakdown and elimination. These conclusions were established by studying the immunogenicity of macrophages at different time intervals after uptake of antigen and correlating it with the intracellular fate of the material. Macrophages after uptake of ^{131}I-MSH rapidly catabolized most of the antigen which was released into the extracellular medium as degraded material, in the form of nonprotein-bound ^{131}I (the justification and limitations for the use of radioactive I as a protein label has been amply discussed in references 23 and 24). The immunogenicity of the cells, however, was not changed by the loss of more than 90% of its original content of antigen. In our previous study using the same cell transfer system, it was shown that the immune response to antigen in macrophages was dose dependent (10); in those experiments, the macrophages containing variable amounts of antigen were immediately transferred after the period of uptake (30–45 min). A 10-fold difference in antigen content as measured before catabolism resulted in a considerable decrease in "priming" activity by the cells. The present experiments have further served to indicate that of the original material taken up in the macrophage, the immunogenic fraction must be represented by less than 10% of it.

Though the present experiments have shown that immunogenicity persisted for long periods of time and was associated with the presence of a relatively stable pool of antigen in the cell, several problems remain unsettled: (a) the exact localization in the cell and the nature of the retained antigen, (b) the way in which the lymphoid cell interacts with this retained antigen, and (c) the relationship between retained antigen and immunological memory.

The stable antigen appears to be localized in an area or compartment of the cell not subjected to the normal digestive process (phagosome-lysosome system) and also accessible to contact with the immunocompetent cell. Whether it reaches this cellular area or compartment after an initial stage through phagosome-lysosome or whether it completely bypasses these organelles is not known. Radioautography at the ultrastructural level of macrophages after uptake of ^{125}I-MSH have indeed disclosed that a large part of the retained antigen was not to be found in lysosomes but was present in cytoplasm in close association with the cell membrane (E. R. Unanue and M. Bedford, material to be published). A suggestion that antigen could be associated with the cell periphery of spleen cells was previously made by Harris using mixed agglutination methods (25). The state of the retained antigen is not well established. Approximately half of the material could be extracted with detergents while the remaining material appeared to be tightly bound to cellular components (26, 27). The antigen which was extractable was heterogeneous in molecular size and included protein of smaller size than the native antigen and degraded material (27). Another point of uncertainty is the association of the retained antigen with RNA. Several investigators have demonstrated that phenol extracts of macrophage contained antigen and RNA (28, 29), but whether this association is important in the retention and immunogenicity of antigen in the live cell is still not clear. We have found that treatment of live macrophages with RNase (50–200 μg/35 $\times$ 10^6 cells) did not lead to a decrease in the amounts of antigen retained or to its immunogenicity.

If the stable antigen is indeed associated with the cell periphery, as it seems, then its interaction with a lymphoid cell would not be difficult to explain. Intimate cell contact with dissolution of the cell membrane and fusion of cytoplasm has been observed between macrophages and lymphocytes (30). On the other hand, antigen could be slowly released from the macrophage to interact with the lymphoid cell. The well-known phenomena of clasmatosis is relevant to this point (31). A third, but less likely, possibility of interaction between antigen and lymphocyte arises from the property that lymphocytes have to penetrate the cytoplasm of cells (32) where they could "contact" intracellular material.

A question of importance is whether there is indeed a continuous priming of lymphoid cell by the retained antigen in the macrophage as has been proposed by some investigators (12, 14). If, in the normal conditions, this phenomena is continuously taking place, then the macrophage must be involved as having an important role in the maintenance of immunological memory, i.e., the capacity to give a secondary type immune response upon an antigenic challenge. Celada has recently demonstrated that memory was a finite process when studied by adoptive transfer in conditions where the lymphoid cells did not have further contact with antigen (33). It may be worthwhile to compare

memory in adoptive transfer of lymphoid cells to animals which have also received macrophages containing antigen. Immunological memory could well be the result of a continuous recruitment of lymphoid cells by retained antigen and persistence of the primed lymphoid "memory" cells. Our experiments with irradiated mice have shown that lymphoid cells could be primed by macrophages a long time after uptake of antigen. However, in a normal animal, as has been previously discussed (34), the retained antigen may become ineffective after the first few days because of processes which inhibit the immune response, such as the presence of some types of antibody.

In conclusion, it appears likely that part of the immunogenic function of macrophages can be explained by their capacity to carry a small percentage of the antigen, in a relatively stable form and isolated from rapid catabolic processes, to foci of potential immunocompetent lymphoid cells. The superior immunogenicity of antigen in macrophages over a comparable amount of soluble native antigen may lie on the greater opportunity that the former has for reaching and interacting with a lymphoid cell. Other reasons for explaining the immunogenicity of macrophages containing antigen, such as change or processing of antigen, are not discarded, but still have to be proved relevant to the normal in vivo conditions. The stimulated macrophages may, however, have another function in immunity quite unrelated to its actual content of antigen, such as the capacity to alter the physiological conditions of lymphoid cells in their neighborhood. This is under present consideration.

SUMMARY

Peritoneal macrophages were cultured for several hours after uptake of ^{131}I-hemocyanin. The cells degraded most of the ^{131}I-labeled protein within 2–5 hr. Their ability to prime lymphocytes of syngeneic mice for a secondary immune challenge remained unchanged for long periods of time despite the loss of more than 90% of the original content of antigen. The persistence of immunogenicity was associated with a small percentage of antigen retained by the cell in a form which was protected from rapid breakdown and elimination.

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