of the unfavourable influence of the preparations studied on the course and outcome of the bacterial infections. But the mechanism of the unfavourable action of MAOI on the organism with a bacterial infection deserves further investigation.

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Persistence of Antigen on the Surface of Macrophages

Mouse peritoneal macrophages can take up and catabolize two different haemocyanins[1,2]. Although in tissue culture experiments most of these materials were broken down in a few hours after uptake, haemocyanin nevertheless elicited the formation of specific antibodies when the macrophages were transferred to syngeneic hosts, and the haemocyanin bound to macrophages retained its immunogenic activity relatively unchanged for several days. In view of the observation that a small percentage of the haemocyanin bound to macrophages remained intact during *in vitro* culture for 72 h, we undertook the experiments described here to determine the localization of the persisting antigen (which would presumably comprise the immunogenic moiety).

In order to study the uptake and catabolism of haemocyanin *in vitro*, macrophages were obtained from the peritoneal exudates of mice (CAF–1, CBA or Swiss Webster) 3 days after each had received an intraperitoneal injection of 1·5 ml. of 10 per cent proteose peptone[1]. The two antigens used were the haemocyanin from giant keyhole limpet (KLH) of molecular weight $7{\cdot}5 \times 10^6$ and the haemocyanin from crabfish (*Maia squinado*) (MSH) of molecular weight around $1{\cdot}5 \times 10^6$. Results with both haemocyanins were comparable. In all experiments $30–50 \times 10^6$ macrophages were exposed to 1,000 μg of MSH or KLH labelled with ^{125}I or ^{131}I (ref. 3; specific activities usually varied from 1 to 10 μCi/μg) in tissue culture flasks containing 6 ml. of Eagle's medium with 5 per cent normal mouse serum. The contents of the flask were constantly stirred with a magnet. After 60–90 min at 37° C, about 0·3 per cent of MSH and 3–5 per cent of KLH was bound to the macrophages. The cells were then washed three times and cultured in media devoid of antigen in either Falcon plastic dishes or in tissue culture bottles, with constant stirring. After several hours the radioactivity (in the form of protein or non-protein bound I) was determined in the culture supernatant and in the cells. In most experiments 10–25 per cent of the radioactivity initially taken up was still in the cells after 4 h, while the remainder was present in the supernatant chiefly as non-protein bound I. Twenty-four to seventy-two hours later the amount of radioactivity bound to the cells was unchanged or only slightly decreased[1,2]. Most of the studies were done on cells cultured for 4–24 h after the uptake of radioactive antigens. During this period the condition of the cells in culture was excellent.

Localization of antigen remaining in the cell after the rapid period of catabolism was studied by electron microscopy autoradiography. Macrophages that had been exposed to MSH labelled with ^{125}I (18 μCi/μg) were cultured for 4 h in medium devoid of antigen. They were then spun into a pellet, fixed in osmium tetraoxide and embedded in methacrylate. Ilford *L4* liquid emulsion was used to coat the slides, and the sections were exposed for 30–60 days. Sixty-six of ninety-five macrophages examined contained autoradiographic grains, although their number varied greatly from cell to cell. Thirty-nine per cent of the grains that were counted were situated on dense organelles (lysosomes); 42 per cent were situated either on small pinocytic vacuoles or in cytoplasm near to vacuoles or lysosomes, and 19 per cent of the grains were located on the cell periphery either on or just contiguous to the cell membrane, or in small villous projections of the macrophage surface (Fig. 1). Most of the macrophages that had grains on their surface also contained grains distributed intracellularly. Thus the capacity to retain material on the surface as well as to engulf it into digestive vacuoles was present in the same cell (see also ref. 1). Although other investigators had previously observed that antigen is retained in the lysosomes of macrophages[4], the finding that antigen is present on the surface many hours after uptake is new. (It should be emphasized that the peritoneal macrophages which we were studying were different from the dendritic reticular cells of the lymphoid follicles which retained antigen on their surface but which did not show pinocytosis[4].)

The presence of antigen retained on the cell surface was also tested in two other ways. Macrophages containing haemocyanin were treated with trypsin (Worthington Biochemicals), on the assumption that trypsin could remove protein antigens exposed on the surface of the cell without significantly disrupting the plasma membrane. Control experiments established that the viability and morphology of 10^7 macrophages cultured in Falcon dishes did not change appreciably after short exposure to trypsin (0·2 mg in 1 ml. of Eagle's medium for 5 min at 37° C). In particular, there were no alterations in the number, size and distribution of the macrophage lysosomes when examined under the ultra-

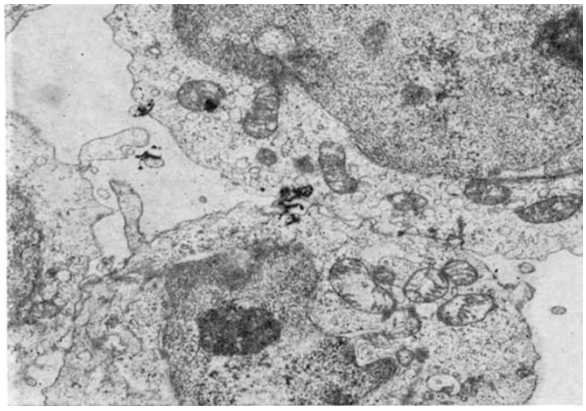

Fig. 1. Electron microscopic autoradiograph of two macrophages labelled with ^{125}I MSH. Note the localization of the grains along the surface and villi of the cells. × 11,700.

violet microscope after exposure to 'Euchrysine'[5]. Macrophages exposed to either MSH or KLH labelled with ^{131}I and cultured in Falcon dishes for 4–72 h were washed five times with large volumes of medium and then treated with trypsin as described. Radioactivity was determined in the supernatant and in the cells detached from the dish with the aid of a rubber policeman. After exposure to trypsin, about 15–25 per cent of the radioactivity present in the cells now appeared in the supernatant, whereas only 1–5 per cent of the radioactivity was released after incubation with medium alone or medium containing ribonuclease (1 mg/ml.) or neuraminidase (500 units/ml.). The radioactive material released by trypsin was 66–80 per cent precipitable in 10 per cent trichloracetic acid and was therefore chiefly protein bound (Fig. 2). Radioactive material removable by trypsin persisted in the cells for as long as 48 h after uptake.

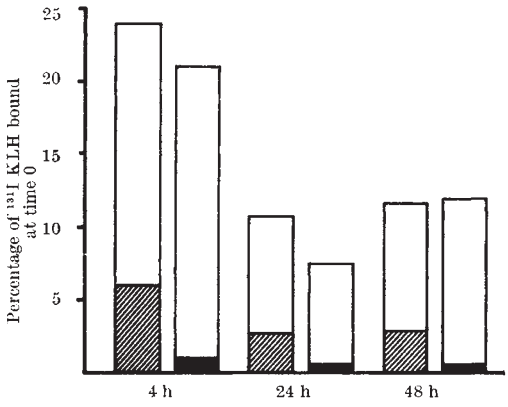

Fig. 2. Macrophages were incubated with KLH labelled with ^{131}I for 1 h at 37° C, washed, resuspended in medium without KLH and plated in Falcon plastic dishes (8×10^6 cells per dish). After 4, 24 or 48 h, the radioactivity was determined in the culture supernatant in order to determine the amount of KLH catabolized. The dishes were then washed five times and incubated with medium alone (controls) or medium containing trypsin. The radioactivity was measured in the supernatant and in the cells (the sum represented the total radioactivity bound to the cells). The amount of radioactive material bound to macrophages is expressed as a percentage of the radioactivity that was present in the cells immediately after uptake (time 0). Each bar represents the average of duplicate cultures. White bars, total radioactivity; hatched bars, radioactivity removed by trypsin; black bars, radioactivity released by medium alone.

The possibility was explored that the antigen on the surface could, in part, derive from material that had been first engulfed in vacuoles. Macrophages which had been cultured for 4–24 h after the uptake of KLH or MSH labelled with ^{131}I were subjected to two treatments with trypsin either consecutively or 4 h apart. Three to five per cent of the radioactivity present in the cells after the first trypsinization was removed by the second treatment regardless of the interval of time. It was concluded therefore that no appreciable amount of new antigen appeared on the surface during the 4 h after the initial trypsin treatment. This suggests that most, if not all, of the antigen removable from the surface had been present there since the initial contact with extracellular antigen and had apparently never been ingested by pinocytosis.

Antigen on the cell surface was also demonstrable by specific binding of antibody to the macrophages. To decrease any non-specific uptake of antibody by macrophages by virtue of the cytophilic properties of immunoglobulin G (IgG) and/or the pinocytic activity of macrophages, two experimental conditions were usually used. First, macrophages were incubated with antibody $F(ab')_2$ or Fab′ fragments. These antibody fragments retained the full antigen-binding capacity of native IgG[6], but had no cytophilic properties[7]. Second, incubation of macrophages with antibody was carried out at 2°–4° C, at which temperature pinocytic and/or phagocytic activities of macrophages were minimal[8].

Rabbit hyperimmune antiserum to KLH was prepared as described previously, and the IgG fraction was obtained by ammonium sulphate precipitation and diethylaminoethyl-cellulose chromatography[9]. As determined by a coprecipitation technique, about 40–50 per cent of the molecules in the various antibody preparations reacted specifically with KLH[6]. Rabbit antibody to MSH purified by the method of Freedman *et al.*[10] was obtained through the courtesy of Dr J. H. Humphrey (National Institute for Medical Research, London).

Table 1. BINDING OF KLH Fab′ ANTIBODY LABELLED WITH ^{131}I TO MOUSE MACROPHAGES INCUBATED *in vitro* WITH KLH LABELLED WITH ^{125}I

Time after uptake*	Antibody preparation† Anti-KLH ^{131}I-Fab′	Anti-BSA IgG	Anti-KLH IgG	^{125}I-KLH bound to 10^7 cells‡	^{131}I antibody bound to 10^7 cells‡	Ratio of antibody to KLH	Specific antibody binding
Hours	μg	μg	μg	μg	μg	μg/μg	μg/μg KLH
4	2·5	500	—	1·38	0·0440	0·0319	0·0256
	2·5	—	500	1·20	0·0076	0·0063	
24	2·5	500	—	1·52	0·0440	0·0289	0·0238
	2·5	—	500	1·45	0·0074	0·0051	
24	2·5	500	—	2·25	0·0274	0·0122	0·0101
	2·5	—	500	3·04	0·0064	0·0021	
	10	2,000	—	3·59	0·0963	0·0268	0·0205
	10	—	2,000	3·13	0·0197	0·0063	
24	10	2,000	—	None§	0·0250	—	
	10	—	2,000	None	0·0200	—	—
	10	2,000	—	1·31	0·0703	0·0537	0·0379
	10	—	2,000	1·46	0·0230	0·0158	

* *In vitro* uptake of KLH was carried out at 37° C for 1 h. After washing, the cells were plated in Falcon dishes (1×10^7 cells/dish) and incubated at 37° C for 4 or 24 h.

† The antibody mixture was contained in 1 ml. of medium which was incubated with 1×10^7 cells at 2° C for 1 h.

‡ Average of duplicate or triplicate cultures.

§ Macrophages were not exposed to KLH.

Results of typical experiments are shown in Table 1. Mouse peritoneal cells were incubated *in vitro* with KLH labelled with ^{125}I at 37° C for 1 h, washed, suspended in fresh medium without KLH and plated in Falcon dishes as described above. After incubation at 37° C for 4–24 h the dishes were cooled to 2° C and washed five times with large volumes of cold Eagle's medium. One ml. of medium containing anti-KLH Fab′ labelled with ^{131}I and 200 times as much unlabelled rabbit anti-bovine serum albumin (BSA) IgG was then added to each dish. Control dishes received one ml. of medium containing anti-KLH Fab′ labelled with ^{131}I and 200 times as much unlabelled anti-KLH IgG, on the assumption that unlabelled anti-KLH IgG in excess would block the specific binding of anti-KLH Fab′ labelled with ^{131}I. After incubation at 2° C for 1 h on a platform rotator and extensive washing with cold Eagle's medium, the radioactivity present on/in the cells was determined. To take into account variation in the number of cells per dish, the amount of cell-bound antibody was calculated per μg of KLH present. As indicated in Table 1, the amount of antibody Fab′ labelled with ^{131}I bound to macrophages containing KLH in the presence of unlabelled anti-BSA IgG was four–six-fold higher than in the presence of unlabelled anti-KLH IgG. No difference was found, however, with macrophages which did not take up KLH. Moreover, 4 or 24 h after the beginning of the *in vitro* culture the amount of antibody Fab′ bound to macrophages containing KLH was identical. A four-fold increase in the concentration of antibody resulted in a three-fold increase in the non-specific uptake of anti-KLH Fab′, whereas the specific binding was doubled. That antibody was chiefly bound to the cell surface was demonstrated by the recovery of 80 per cent of the radioactivity in the supernatant after treatment with trypsin of KLH-containing peritoneal cells incubated with anti-KLH Fab′ labelled with ^{131}I.

Similar data were obtained with suspensions of macrophages containing KLH incubated with anti-KLH $F(ab')_2$ labelled with ^{131}I. When MSH was used, two to three times more anti-MSH purified antibody labelled with ^{131}I was bound to macrophages containing MSH than to normal macrophages. For example, 0·015 μg of anti-MSH IgG bound specifically to 9×10^6 macrophages containing 1·1 μg of MSH 24 h after uptake.

We have thus demonstrated the presence of antigenic material on the surface of macrophages incubated *in vitro* with haemocyanins. This material persisted on the cell surface for up to 72 h without being incorporated into vacuoles, and thereby escaped the normal catabolic breakdown associated with its presence in pinolysosomes. The reasons for the persistence of antigenic material on the cell surface of macrophages are not known. Assuming that KLH is retained in its associated form (molecular weight: $7·5 \times 10^6$ (ref. 11)), an average of 10^5 molecules of KLH were taken up *in vitro* by one single macrophage. After incubation for a few hours at 37° C, only $1·5 \times 10^4$ molecules of KLH were still retained by the cell, 20 per cent of which (or 3×10^3 molecules) were present on the cell surface. About 6×10^4 molecules of anti-KLH antibody specifically reacted with such a macrophage (assuming a molecular weight of 46×10^3 for rabbit Fab'). In other words, of 1,000 KLH molecules to which a macrophage was exposed *in vitro*, thirty-three were taken up, but only one molecule remained attached to the cell surface and this reacted with twenty molecules of antibody. (In extreme antibody excess in solution, one molecule of free associated KLH is saturated by 200 molecules of IgG antibody[12].) With MSH, one of every 10,000 molecules to which a macrophage was exposed remained bound to the surface. The part played by the antigenic material persisting on the macrophage surface in the induction of the immune response has not yet been established. Considering the availability of this material to antibody *in vitro*, however, it is tempting to speculate that a similar interaction can occur *in vivo* between antigenic molecules on a macrophage surface and antibody-like receptors of antigen-sensitive cells.

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Thymus Dependent Mesothelial Proliferation after Antigenic Stimulation

Because of its membranous nature, the greater omentum of the mouse is a convenient tissue for serial studies of the cellular events in an immune response. Using mice it was found that after intraperitoneal stimulation with sheep erythrocytes the number of milk spots on the omentum was markedly increased[1]. These milk spots represent accumulations of varying numbers of lymphoid cells and macrophages. By a modification of the local haemolysis in gel technique it was shown that large milk spots produced haemolytic antibodies against sheep erythrocytes[1]. After repeated stimulation, clusters of plasma cells could be identified in the vicinity of relatively large milk spots. In some experiments the omenta were explanted into tissue culture, and continued to produce antibody *in vitro* for more than 60 days[2].

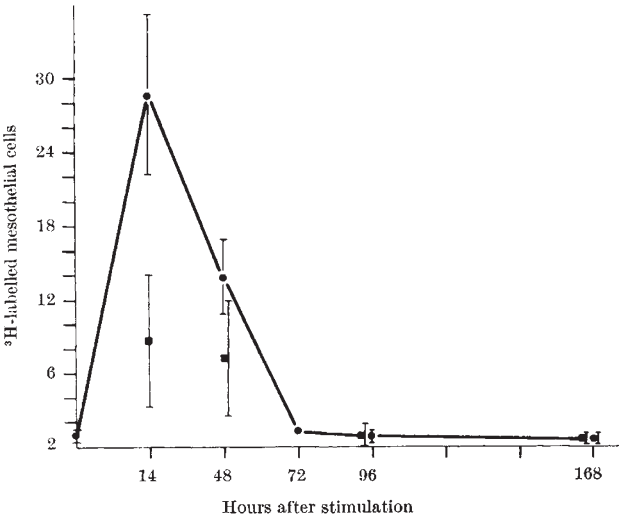

Fig. 1. Means and standard deviations of mesothelial cell proliferation in the greater omentum after intraperitoneal stimulation with sheep erythrocytes in neonatally thymectomized NMRI mice (■) and sham-operated (●——●) NMRI mice. Ordinate: percentages of labelled mesothelial cells after *in vitro* incubation with ^{3}H-thymidine. Abscissa: hours after intraperitoneal antigenic stimulation.

In recent experiments the mitotic activity of the cells on the omentum was investigated[3]. After labelling with ^{3}H-thymidine, autoradiographs of the omenta were prepared, and these demonstrated unequivocally that not only lymphoid cells but also mesothelial cells responded to antigenic stimulation by a striking increase in ^{3}H-thymidine uptake. Antigen-stimulated cell proliferation may be non-specific in the sense that it is not restricted to those cells which become specifically sensitized or eventually produce specific antibody[4]. The mesothelial cell proliferation which was observed in our previous study presumably represents such a non-specific component of the immune response. Neonatal thymectomy considerably reduces immunological reactivity (for review, see ref. 5). Also, in the absence of the thymus the inflammatory response to subcutaneously implanted cotton pellets is reduced[6]. The thymus may influence the immune response in two different ways: first, by providing "thymus derived" cells as essential collaborators[7–11] and, second, by influencing (*a*) the specific, and (*b*) the nonspecific cell proliferation after antigenic stimulation. The existence of the latter mechanism may conveniently be tested by assessing the effect of thymectomy on the antigen-stimulated proliferation of the mesothelial cells