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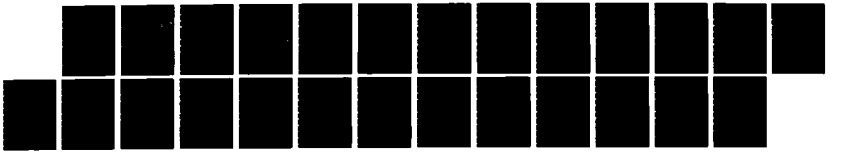

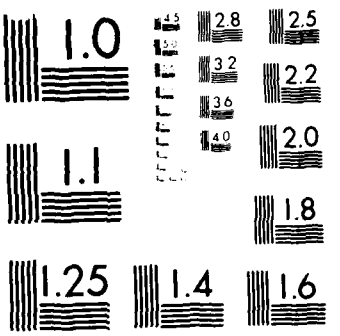

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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

Non-toxic synthetic adjuvants are under prime consideration for use in increasing the immune response of human beings. Three of microbial origin are the muramyl di-peptides (N-acetyl-muramyl-L-alanyl-D-isoglutamine and analogs, termed MDP), polyadenylic acid-polyuridylic acid complexes termed poly A·poly U) and the recently isolated monophosphoryl lipid A (termed MPL). While each of these has been demonstrated in animals to be active in increasing the immune response when given with the antigen, each also has been found to suppress this response when given one to several days before antigen. The enhancing actions of MDP and poly A·poly U have been well characterized. However, characterization of the suppressive phenomenon has been minimal, but is important to gain a responsible understanding of how these adjuvants regulate the immune response non-specifically. Accordingly, the experiments proposed during the tenure of this contract were undertaken to further our knowledge of how each of these adjuvants activate the suppressive arm of the immune response. The data are submitted in the form of four preprints and one reprint and

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permit the following main conclusions:

POLY A · POLY U

1. Poly A·poly inhibited antibody forming cells non-specifically when given 1-4 days before antigen, whereas poly I·poly C inhibited when given 1-6 days before antigen.
2. This suppression could be expressed in in vitro experiments by addition of surprisingly either T cell rich, B cell rich or adherent cell populations to their syngeneic normal cell counterparts, suggesting an unidentified cell may be contaminating each.
3. To determine whether an NK cell was contaminating the above 3 populations and was responsible for suppression, NK activity was removed with anti-asialo GM1 antibody without affecting the magnitude of the suppression.
4. Suppressive activity for both humoral and cell mediated immunity (MLR) was found and characterized in the serum of mice injected with poly A·poly U after 90 minutes.
5. Poly A·poly U increased non-specific resistance to Streptococcus pneumoniae and Pseudomonas aeruginosa when given 1-2 days before challenge with these microorganisms, despite the presence of antibody suppressing activity in the spleen at this time.

MURAMYL DI-PEPTIDES

1. A single injection of MDP either ip or iv, 1-2 days before antigen inhibited antibody forming cells by approximately 50%. This suppression lasted from 4-14 days with much individual variation.
2. Using derivatives of MDP it was shown that the muramyl grouping was not necessary for suppressive activity. The addition of an n-butyl ester grouping to the terminal carboxyl of the glutamine moiety of MDP did not increase the capacity to induce suppression.
3. Suppression could be transferred to syngeneic recipient mice with both adherent and non-adherent spleen cells. T cells were found to be the effector cell in the latter population.
4. Unlike poly A·poly U, MDP did not induce suppressive activity in the serum 90 minutes after injection.
5. Interleukin I activity was depressed 24 and 48 hr after MDP injection, while Il-2 activity became depressed later at 72 hr.
6. It was hypothesized that MDP initiates suppression in the macrophage population in the form of decreased Il-1 production, which in turn depressed Il-2 levels. The net result was a decrease in numbers of antibody forming cells.

MONOPHOSPHORYL LIPID A

1. A non-toxic monophosphoryl lipid A (MPL, Ribi) isolated from endotoxins of Gram negative bacteria was shown to exert an adjuvant action on both the helper and suppressor branches of the immune response. Thus, toxicity is not a requirement for the adjuvant action of bacterial endotoxins.
2. MPL restored antibody production in aging mice and in the endotoxin low responding mouse strains C3H/HeJ and C57B110/ScN. In addition, MPL induced suppression in the C3H/HeJ strain.

GENERAL

1. Poly A·poly U, MDP and LPS increased phagocytosis in macrophages from young mice, but appeared to suppress this activity in aging virgin mice.
2. Aging breeder mice on the other hand were activated to increased phagocytosis similar to young mice, suggesting hormonal factors may control certain reactivities to adjuvants.

Differential Responses to Adjuvants of Macrophages from Young Virgin, Aging Virgin and Aging Breeder Mice

by

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Abstract

Age related differences in macrophage responsiveness to adjuvants seen previously with thioglycollate induced cells also were found with resident macrophges. Thus, activation of phagocytosis by lipopolysaccharide, polyadenylic-polyuridylic acid complexes and muramyl dipeptides occurred when exposed to macrophages removed from young but not aging C58 and C3H/He mice. However, breeding status differences were observed in that aging virgin mice were unresponsive to these adjuvants, while aging breeder mice responded similar to young virgin mice. Analysis of any changes in membrane phospholipids was carried out to determine their association with the age related and breeding status differences observed. Significant differences occurred in ^{32}P labelling of phosphatidyl inositol between unstimulated macrophages from young and aging C58 mice. No differences were evident, however, after LPS stimulation. Analysis of macrophage plasma membranes from young and aging mice for cholesterol revealed significant age related differences in their ability to undergo increases in cholesterol content after LPS activation.

Introduction

Recently we reported our studies of the effect of age on the responsiveness of macrophages from four strains of mice to several synthetic adjuvants (Petrequin and Johnson, 1984). In contrast to macrophages from young mice, macrophages from aging C58, Balb/c and C3H/He mice failed to respond with increased in vitro phagocytosis or tumor cytotoxicity following exposure to the adjuvants polyadenylate-polyuridylate complexes (poly A·poly U), muramyl di-peptide (MDP) or lipopolysaccharide (LPS). LPS and poly A·poly U also failed to increase hexose monophosphate shunt activity in aging mice of the C58 and C3H/HeN strain. A surprising finding was the reversal of response

patterns observed between aging virgin and aging breeder C58 mice in the chemiluminescence and hexose monophosphate shunt assays. Thus, macrophages from aging breeding mice were capable of responding like young virgin mice to PMA and LPS activation respectively, whereas those from aging virgin mice did not respond. In this earlier study, macrophages elicited with thioglycollate broth were used. However, evidence has accumulated indicating that resident macrophages may react differently than elicited cells (Morahan, 1980).

Accordingly, this manuscript reports our investigation of the effects of age and breeding on macrophage responsiveness with experiments designed to answer the following questions: (a) do aging resident macrophages differ from thioglycollate stimulated macrophages in lacking responsiveness to potent adjuvants? (b) are the differences between aging breeder and aging virgin mice found previously in their response to LPS in the chemiluminescence and to PMA in the hexosemonophosphate shunt assays, to be found in other macrophage functions and strains of mice and with other adjuvants? (c) is the lack of responsiveness of aging virgin mice associated with changes in the membrane phospholipids and/or cholesterol of aging as compared to young mice?

Materials and Methods

Mice: Three strains of mice (C58, C3H/He, and Balb/c) were raised in our inbred mouse colonies. The C57Bl/6J and B6D2F_1 mice were purchased from Charles River Laboratory (Baltimore, MD).

Cells: Adherent peritoneal exudate cells, PEC, were prepared as follows: PECs were obtained by washing with Hanks balanced salt solution (HBSS) (Gibco, Grand Island, NY) the peritoneal cavity of mice uninjected (resident) or injected 3 days previously with 2.5 ml of 1M thioglycollate broth (Difco, Detroit, MI) (elicited). Cells from 2-3 mice per age group were pooled, centrifuged at 150xg and resuspended in H-3 media (M.A. Bioproducts,

Walkersville, MD) with Limulus negative, 10% fetal calf serum (FCS) (Reheiss, Phoenix, AZ) and counted. The young cells were shown to be 95% and the old 85% positive in nonspecific esterase staining. All experiments were repeated at least three times.

Activating agents: Lipopolysaccharides, LPS, were a gift from Dr. A. Nowotny, Univ. Penn., Philadelphia. They were phenol-water preparations from Salmonella minnesota smooth and rough strains and used at a final concentration of 10 µg/ml in HBSS.

Polyadenylate and polyuridylate (Miles Laboratories, Kankakee, IL) complexes were prepared by mixing equal volumes of the two polynucleotides at concentrations of 2 mg/ml in 0.15M PBS at room temperature for 30 min, the solution diluted to 1 mg/ml, and used at 50 µg/ml final complex concentration. Muramyl dipeptide (Pasteur) was a gift from Dr. L. Chedid, Paris, France. A 1 mg/ml solution in HBSS was brought to pH 7.2 with 0.01% triethylamine (Sigma, St. Louis, MO) and used at 5 µg/ml for activation.

Phagocytosis Assay (Dunn, Eaton, Lopatin, Tryor, Entire and Papermaster, 1981): PECs were diluted to 1 x 10^6 cells/ml in H-3 medium and 30 µl added to wells of a 10 hole Bellco microslide chamber (Bellco Glass, Inc., Vineland, NY). Five microliters of activating agent were then added to a final concentration of LPS, 10 µg/ml; poly A·poly U, 50 µg/ml; MDP, 5 µg/ml. PBS was added to control wells. Twenty microliters of fluorescent bead suspension (Fluoresbrite #9847, Poly Sciences, Inc., Warrington, PA), diluted 1:500 in .05M PBS pH 7.1, were added to each well. The microchamber slides were then incubated 1½ hours at 37°C in 5% CO_2. Nonadherent cells and extracellular beads were rinsed off by gently pouring saline across the detached slide held at an angle, and the cells fixed with 1 drop of 1% glutaraldehyde.

The number of cells in 100 containing beads (% phagocytic cells) and the number of beads in those 100 cells were counted under a fluorescent microscope and the phagocytic index calculated as:

$$\text{Phagocytic Index} = \frac{\%\ \text{phagocytic cells X number beads/100 cells}}{1000}$$

Phospholipid Labelling with [^{32}P]-Orthophosphoric acid (Zeleznikar, Quist and Drewes, 1983): The preparation of macrophages for labelling in suspension utilized 5 x 10^6 PEC in 12 ml of medium with FCS. They were plated on 250 ml flasks containing BHK microexudate surfaces and incubated at 37°C in 5% CO_2 for 24 hr, and removed by centrifugation at 200xg for 10 min. The cells were then washed with PBS and counted. They were centrifuged again and resuspended to a 2 ml volume in labelling media (10mM Hepes pH 7.4, 125mM NaCl, 5mM KCL, and 0.5mM KH_2PO_4, 1.0mM $MgCl_2$, 1.4mM $CaCl_2$, and 10mM glucose) at a concentration of 1.0 x 10^7 cells/ml.

[^{32}P]-orthophosphoric acid 5mCi (Amersham-Searle, Arlington Heights, IL) was added to the macrophages to a final concentration of 50 µCi/ml. Cells were incubated for various times during the time course experiments or for 60 min otherwise. The reaction was stopped by micropipetting 0.25 ml of the reaction mixture into 2.0 ml of extraction solvent, chloroform:methanol:hydrochloric acid ($CHCl_3$:CH_3OH:HCl) 20:40:1 with .01% dithiothreitol (Sigma, St. Louis, MO).

Labelling of adherent cells utilized 5.0 ml of 1.0 x 10^6 cells/ml added to 50 ml flasks (Kontes, Evanston, IL) containing BHK microexudate surfaces. After overnight incubation, the H-3 media was removed and cells washed twice with PBS. Five milliliters of labelling media were added and the reaction was initiated by adding [^{32}P]-orthophosphoric acid so that the final concentration was 50 µCi/ml. The reaction was stopped by adding 1.0 ml of 10mM EDTA in .15M

PBS and putting the cells on ice. After removing the cells and centrifuging at 400xg, 2.0 ml of extraction media was added.

Phospholipid Extraction: Phospholipids (PL) were extracted by a modification of the method of Bligh and Dyer (1959). After adding the labelled cells to 2.0 ml of $CHCl_3$:CH_3OH:HCl (20:40:1) for extraction, the tubes were covered, put on ice, and allowed to stand for 30 min. Choloroform (0.8 ml) and water (0.7 ml) were then added, and the tubes were vortexed and centrifuged for 10 min at 1000xg at 4°C. The aqueous phase was removed, and the chloroform phase was washed twice with 2.0 ml of 0.1N HCl. A 1.0 ml aliquot of the chloroform phase was dried under N_2 and stored overnight at -20°C.

Thin Layer Chromatography and Audioradiography: The dried lipid extracts were dissolved in 45 µl of $CHCl_3$:CH_3OH (2:1), 20 µl aliquots were spotted on silica Gel 60 plates (20x20 cm, 0.2 mm thick) (Scientific Products, McGraw Park, IL) and 20 µl were put in a 15 ml pyrex tube and dried under N_2 for phosphate determination, as described below. The phospholipids were separated by a solvent system (Schacht, Neale and Agranoff, 1974), which was chloroform:methanol:methylamine (65:35:10), for 2-3 hours. Radioactive phospholipids were detected by exposing the dried plates to Kodax X-OMAT R film for 2 days. The ^{32}P labelled phospholipids were scraped from the plate and the radioactivity was determined by liquid scintillation detection in Tritosol scintillation fluid. The identity of the PL spots was determined by spotting standard preparations of individual phospholipids (Sigma, St. Louis, MO) on each TLC plate.

Phosphate Analysis: The phospholipid content of the other 20 µl portion of PL extracts was estimated by phosphate analysis as described by Bartlett (1959). One milliliter of water and 0.5 ml of 5N H_2SO_4 were added to the

pyrex tubes containing the dried 20 µl aliquots from the total PL extracts described above. The tubes were then heated for 3 hours in a 150°C oven, 0.025 ml of 30% H_2O_2 added, and the samples heated again for 1.5 hours at 150°C. The samples were cooled and 0.2 ml of 5% ammonium molybdate and 0.2 ml of ANS reagent (1 mM 1-amino-2-napthol-4 sulfonic acid and 4 mM sodium sulfite in 15% sodium bisulfite) were added. The tubes were heated in a boiling water bath for 7 min and the optical (OD) density was read on a Beckman DB-G spectrophotometer at 680 nm.

The micrograms of total PL were estimated by assuming there were 25 µg of phospholipid (PL)/µg phosphate (P) permitting the use of the following formula:

µg PL = µmoles x 30 µg P/µmole P x 25 µg PL/µg P

The number of µg of individual PL's was determined by knowing the per cent each PL subclass contributed to the total PL extract as was determined in Table 4 of the Results. The specific activity of each phospholipid in a sample was calculated by dividing the counts per 5 min of the individual PL spots by the µg of individual PL. Because of the short half-life of ^{32}P, there was variation in the ^{32}P counts incorporated from experiment to experiment. Consequently, the data usually represented the per cent each PL subclass contributed to the sum of the specific activities of all the PL subclasses.

Results

Comparison of the activation of phagocytosis by resident macrophages from young and aging mice by LPS, Poly A·Poly U and MDP is shown in Table 1. These results compare favorably with what had been found earlier with thioglycollate elicited cells (Petrequin and Johnson, 1984) in that resident cells from young mice of the C58 and C3H/He strains were activated significantly (2 fold),

whereas those from aging mice were not. Resident macrophages from the Balb/c strain exhibited a similar pattern of lessened response in the aging mice, but due to the higher than usual variation in these 3 experiments did not attain the degree of significance found previously with thioglycollate induced macrophages. Resident cells from the $B6D2F_1$ strain also did not show statistically significant age related differences in their ability to be activated, but the differences exhibited between young and aging resident cells were greater than those seen with thioglycollate elicited cells. In agreement with the findings of Karnovsky, Drath and Harper (1975), the unstimulated baseline phagocytic activity was approximately two-fold higher for resident cells than those elicted by thioglycollate.

To determine whether resident macrophages from aging female C58 mice exhibited the differences between virgins and breeders seen previously with thioglycollate induced cells, the ability of resident macrophages from such mice to be stimulated in phagocytosis by LPS and MDP was compared. A similar pattern of response as seen previously (Petrequin and Johnson, 1984) was observed (Table 2). Resident cells from aging breeder mice were activated 2 fold which was similar to the response of macrophages from young virgins and significantly different from that of cells from aged virgin mice. Also noteworthy, was the finding that the unstimulated activity of cells from aged breeders was similar to those from aged virgins, being 2 fold higher than that of young virgin mice. Of interest was the similarity in responses to both MDP and LPS, suggesting the differences between breeder and virgin mice may be a general response pattern to adjuvants.

To extend the legitimacy of these differences with another strain and a third adjuvant, poly A·poly U, thioglycollate elicited macrophages from aged breeder Balb/c mice were compared to those from young and aged virgin Balb/c

mice for their ability to be activated in phagocytosis by LPS and poly A·poly U (Table 3). Cells from aged breeder Balb/c mice were activated approximately 2 fold as were those from young virgin Balb/c mice, while those from aged virgin mice were not activated. Thus, as with the C58 strain, the response of the aged breeders was closer to that of the young virgins and significantly different from that of aged virgins. The unstimulated activity of the aging breeders also was similar to that seen with the C58 strain in that phagocytosis by cells from aging virgins was about 2 fold higher than that of young virgin mice.

Experiments were initiated to determine the mechanism of these age related and breeding status differences. Inasmuch as phospholipid turnover has been shown to be one of the early events in cellular activation, changes in these lipids were investigated first. Since earlier studies had demonstrated that the addition of LPS to macrophages increased the [^{32}P]-orthophosphoric acid labelling of their minor phospholipid subclasses (Graham, Karnovsky, Shafer, Glass and Karnowsky, 1967; Ogmundsdotter and Weir, 1979), a comparison of the abilities of macrophages from young and aging mice to undergo this increased turnover of phosphorus groups after LPS activation was made.

Initially, the phospholipid content of the individual subclasses was determined in adherent cells from young and aging mice. The percentage that each phospholipid subclass contributed to the whole chloroform:methanol extract of macrophages from young and aging C58 mice is compared in Table 4. A difference in content of only phosphatidyl inositol (PIO) was found, in that PIO levels were significantly higher in nonactivated cells of aging mice as compared to those from the young. The activation of macrophages from young C58 mice by LPS was found to be associated with an increase in the content of

PIO in these cells. In further tests no age related differences were found in three other murine strains, C3H/He, Balb/c, and $B6D2F_1$.

The ability of macrophages from young and aging mice to incorporate ^{32}P in suspension cultures into total phospholipid extracts also was studied. No age related differences were found in the ability to incorporate ^{32}P into C58 cells. A similar negative finding was made when testing the C3H/He, Balb/c, and $B6D2F_1$ strains.

These experiments were extended to determine whether there was any effect of age on ^{32}P uptake into individual phospholipid subclasses of unstimulated cells in suspension cultures from young and aging virgin and aging breeder C58 females. The results are shown in Table 5. Two phospholipid subclasses, phosphatidyl choline and PIO, were found to be labelled statistically differently between young and aging virgins. Of particular significance was PIO, which labelled to a 2 fold greater extent in macrophages from young mice. This was not entirely explained by the lower content of PIO in these cells (Table 4). Interestingly, cells from aging breeder C58 mice once again were intermediate between those from young and aging virgins in their incorporation of ^{32}P into PIO, in agreement with our finding that aging breeders are more like young virgins in their responsiveness.

The effect of LPS on the incorporation of ^{32}P into the individual PL subclasses also was examined in macrophges from young and aging mice of the C58 strain (Table 6). Like Karnovsky et al.'s (1975) and Ogmundsdotter and Weir's (1979) previous findings, a significant increase in labelling of the minor phospholipids, phosphatidyl serine, and PIO, occurred with LPS stimulation of cells from young mice. Although activation of macrophages from aging mice showed some increased labelling of these fractions, it was not statistically significant from control values. Statistical analysis, by a

paired t test, also showed there was no significant difference between young and aging mice in their ability to be activated by LPS in the ^{32}P labelling of minor phospholipids.

Since differences were not found in the turnover of total phospholipid by young and aging macrophages after LPS stimulation, changes in cholesterol content were investigated. Previously, Schlager and Meltzer (1981) had demonstrated 2-3 fold increases in cholesterol content in macrophage plasma membranes after lymphokine activation; consequently, macrophage plasma membranes were prepared according to the procedure of Burnette and Till (1971) and the cholesterol composition of such preparations from LPS stimulated and unstimulated macrophages from young and aging C58 mice were compared. The results (Table 7), revealed age related differences in the ability of this strain to respond to LPS stimulation with an increase in cholesterol. A significant increase in cholesterol content after LPS activation was found in membranes from young but not from aging C58 mice by paired t analysis.

Discussion

Age related differences in phagocytosis in response to adjuvants that were reported previously with thioglycollate elicited macrophages (Petrequin and Johnson, 1984) occurred as well when using resident cells from the C58 and C3H/He strains. In addition, the $B6D2F_1$ strain, as in previous results with thioglycollate induced cells, did not exhibit significant age related differences with resident cells.

Also, the breeding status differences that were evident with thioglycollate elicited cells in our earlier study (Petrequin and Johnson, 1984) remained when resident cells from the C58 and a second strain, Balb/c, were activated in phagocytosis. Their extension to other adjuvants and additional strains of mice suggests the general thesis that macrophages from

aged breeder mice respond more like those of young virgins and significantly different from cells of aging virgins. These data offer additional support for the hypothesis that breeding prevents or delays at least some age related changes in immune responsiveness. Speculation as to possible hormonal involvement is fueled by the fact that during pregnancy and early lactation, estrogen and progesterone are at higher levels than in a nonbreeding state (Johnson and Everitt, 1978). In addition, recent studies have revealed adjuvant effects of prolactin on immunocompetent cells (Spangelo, Hall and Goldstein, in press, 1985). Consequently, the increase in circulating hormones during breeding and nursing, when considered with the evidence that these hormones under certain conditions can increase the immune response (Kenny, Pangburn and Trial, 1976; Krzych, Strausser, Busaler and Goldstein, 1978; Strausser, Fiore and Belisle, 1983) as well as the phagocytic function of the RES (Nicol and Vernon-Roberts, 1965), suggests them as likely candidates for future study. In agreement with the functional results, the experiments measuring ^{32}P incorporation by unstimulated cells from aging breeders showed that the amount of label incorporated into PIO was intermediate between the values for the young and aging virgins. A cause and effect relationship remains to be established.

Although the basal phagocytic activity of cells from aging breeder mice was similar to that of cells from aging virgin mice (i.e. elevated in comparison to that of cells from young mice), the addition of adjuvants to the cells from aging breeders increased further their response, such that the fold increase in response of the cells from aged breeders was similar to that seen with macrophages from young mice. This fact renders less plausible the possibility that the adjuvants could not activate macrophages from aging mice

because of an already acquired state of maximal activity following a lifetime of microbial exposure.

Preliminary investigation into the mechanism of the age related and breeding status differences observed in macrophage function involved comparison of the ability of cells from young and aging mice to undergo early changes in membrane lipid composition. Investigation of increases in phospholipid turnover and cholesterol composition of macrophages after stimulation with LPS revealed significant age related differences in the case of the latter. The 1.8 fold increase in cholesterol content seen with the young macrophages was slightly lower than the 2-3 fold increases described by Schlager and Meltzer (1981) who studied young cells from another mouse strain which were induced by PBS instead of thioglycollate, and who prepared their plasma membranes differently and stimulated with lymphokine. With our procedures, we were unable to detect a difference in labelling of the minor phospholipids of macrophages from young and aging mice after activation with LPS. Differences in ^{32}P labelling of the PIO fraction of unstimulated macrophages from young and aging C58 mice, however, were observed. A hypothesis for future study is whether the age related differences in activation by LPS may lie in the inability of macrophages from aging mice to undergo an increase in cholesterol content after stimulation.

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TABLE 1: Activation of phagocytosis by LPS, poly A:poly U, and MDP in resident macrophages from young and aging mice.

Strain	Age	% phagocytic cells without adjuvant	# of beads in 100 cells without adjuvant	PI[a] without adjuvant	Ratio of Phagocytic Indices[b]		
					+LPS / -LPS	+poly A:poly U / -poly A:poly U	+MDP / -MDP
C58	4 mo	51 ± 5	60 ± 12	4.4 ± 1.3	2.7 ± 0.6	2.0 ± 0.4	2.2 ± 0.4
	12 mo	75 ± 2	159 ± 20	10.5 ± 2.6	0.6 ± 0.1 [c]	0.7 ± 0.3 [c]	1.1 ± 0.1 [c]
BALB/c	6 mo	37 ± 5	56 ± 5	4.9 ± 3.4	2.7 ± 0.6	1.9 ± 0.3	2.3 ± 1.0
	27 mo	49 ± 7	89 ± 7	7.6 ± 4.3	1.3 ± 0.2 [c]	0.7 ± 0.2 [c]	1.3 ± 0.4
C3H/He	5 mo	84 ± 6	102 ± 10	4.4 ± 1.0	2.3 ± 0.7	1.4 ± 0.3	2.6 ± 0.5
	15 mo	68 ± 3	146 ± 12	9.7 ± 3.2	0.5 ± 0.1 [c]	0.7 ± 0.2 [c]	1.0 ± 0.4 [c]
B6D2F$_1$	7 mo	52 ± 8	126 ± 6	4.0 ± 2.0	2.6 ± 1.0	1.3 ± 0.1	2.4 ± 0.8
	25 mo	60 ± 7	131 ± 28	8.3 ± 4.2	0.6 ± 0.2	0.7 ± 0.5	0.7 ± 0.2

[a] PI = Phagocytic Index = $\frac{\%\ \text{phagocytic cells} \times \#\ \text{beads in 100 cells}}{1000}$

[b] The mean ± S.E.M. of at least 3 experiments.

[c] p value < .05 in paired t analysis for age related differences.

TABLE 2: Activation of phagocytosis by LPS and MDP in resident macrophages from young and aging virgin and aging breeder female C_{58} mice.

Status	Age	PI[a] without adjuvant	Ratio of Phagocytic Indices[b]	
			$\frac{+LPS}{-LPS}$	$\frac{+MDP}{-MDP}$
Virgin	4 mo	4.4 ± 1.3	2.7 ± 0.6	2.2 ± 0.4
Breeder	12 mo	10.7 ± 2.4	1.7 ± 0.1 [c]	2.0 ± 0.3 [c]
Virgin	12 mo	10.5 ± 2.6	0.6 ± 0.1	1.1 ± 0.1

[a] P.I. = Phagocytic Index = $\frac{\% \text{ phagocytic cells} \times \# \text{ beads in 100 cells}}{1000}$

[b] The mean ± S.E.M. of at least 3 experiments.

[c] p value < .05 in paired t analysis for breeding status differences.

TABLE 3: Activation of phagocytosis by LPS and Poly A:Poly U in thioglycollate elicited macrophages from young and aging virgin and aging breeder female BALB/c mice.

Status	Age	PI[a] without adjuvant	Ratio of Phagocytic Indices[b]	
			$\frac{+LPS}{-LPS}$	$\frac{+Poly\ A:Poly\ U}{-Poly\ A:Poly\ U}$
Virgin	6 mo	1.3 ± 0.4	2.7	2.3
Breeder	27 mo	4.4 ± 1.2	2.3	2.1
Virgin	27 mo	6.2 ± 1.2	1.2[c]	1.1[c]

[a] PI = phagocytic Index = $\frac{\%\ \text{phagocytic cells} \times \#\ \text{beads in 100 cells}}{1000}$

[b] The mean ± S.E.M. of at least 3 experiments.

[c] p value < .05 in paired t analysis for breeding status differences.

TABLE 4: Bartlett assay on adherent peritoneal exudate cells from young and aged C_{58} mice.

Phospholipid	Percentage of Total Phospholipid[a]			
	Young	Aged	Young + LPS[b]	Aged + LPS[b]
Phos. Ethanolamine	19.3 ± 1.1	20.8 ± 2.3	18.3 ± 1.5	25.7 ± 1.8
Cardiolipin	4.1 ± 0.2	4.3 ± 0.4	4.3 ± 0.1	4.2 ± 0.5
Phos. Choline	49.0 ± 2.3	45.8 1 2.8	48.0 ± 1.2	35.0 ± 6.5
Sphingomyelin	7.8 ± 1.1	6.6 ± 1.3	7.2 ± 1.5	6.5 ± 1.5
Phos. Serine + Phosphatidic Acid	16.0 ± 3.0	17.2 ± 4.1	17.4 ± 2.9	12.3 ± 4.1
Phos. Inositol	7.4 ± 0.6	10.2 ± 0.9[c]	9.4 ± 0.7[c]	16.7 ± 6.0

[a]Mean percentages ± S.E.M. of µg individual PL/µg total PL of $CHCl_3$:methanol extracts of C_{58} macrophages from young and aged virgin mice (5 experiments).

[b]Macrophages were incubated with 10 µg/ml LPS for 60 min.

[c]$p < .05$ as compared to the data of cells from young mice.

TABLE 5: Proportion of ^{32}P incorporation into individual phospholipid subclasses by young and aging C_{58} macrophages labelled in suspension.

Phospholipid	Percentage of Incorporation into Subclasses of PL[a]		
	Young Virgin	Aged Virgin	Aged Breeder
Phos. Ethanolamine	16 ± 6	14.5 ± 4	21 ± 3
Cardiolipin	4.8 ± 1	4.3 ± 1	3.2 ± 0
Phos. Choline	31 ± 3	45 ± 10[b]	42 ± 2[b]
Phos. Serine + Phosphatidic Acid	38 ± 14	26 ± 12	17 ± 1[b]
Phos. Inositol	22 ± 6	12.8 ± 5[b]	17 ± 0

[a]Calculated as a ratio of cpm/min/µg PL of individual PL : Sum of cpm/min/µg PL of all PL's where samples were counted over 5 min ± S.E.M. from 3 experiments after a 60 min. incubation of ^{32}P with macrophages from young (4 mo) and aged (12 mo) C_{58} mice.

[b]p value < .05 in paired t analysis for age related differences.

TABLE 6: ^{32}P incorporation into phospholipid subclasses after activation of C_{58} macrophages with LPS.

Phospholipid	Age	Specific Activity[a]	
		-LPS	+LPS[b]
Phos. Ethanolamine	4 mo	29 ± 7	44 ± 16
	12 mo	21 ± 3	30 ± 17
Phos. Choline	4 mo	89 ± 15	108 ± 30
	12 mo	94 ± 19	123 ± 46
Phosphatidic Acid + Phos. Serine	4 mo	191 ± 50	289 ± 52[c]
	12 mo	147 ± 44	174 ± 45
Phos. Inositol	4 mo	154 ± 25	232 ± 24[c]
	12 mo	117 ± 24	169 ± 34

[a] Mean specific activity (cpm/ugPL) ± S.E.M. from 5 experiments.

[b] LPS at 10 ug/ml was present during 60 min ^{32}P labelling. Virgin mice used.

[c] Significantly greater than nonactivated cells. Criterion for significance was $p < .01$ as determined by the t test.

TABLE 7: Cholesterol content of whole cells and plasma membrane isolates of macrophages from young and aging C_{58} mice after activation with LPS.

Source	Age	Cholesterol[a] (µg/µg P)	
		Control	+LPS[b]
Whole Cells	4 mo	.27 ± .07	.23 ± .05
	12 mo	.21 ± .09	.17 ± .12
Plasma Membrane Isolates	4 mo	.11 ± .04	.19 ± .02[c]
	12 mo	.17 ± .05	.22 ± .08

[a]Mean cholesterol values ± S.E.M. of 3 experiments using $CHCl_3$:methanol (1:2) extracts.

[b]Cells were incubated with an R7 LPS at 10 µg/ml for 18 hours before extraction.

[c]$p < .05$ in paired t analysis of ability of young and old cells to be activated.

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