

Phagocytosis, Peritoneal Influx, and Enzyme Activities in Peritoneal Macrophages from Germfree, Conventional, and Ex-Germfree Mice

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Peritoneal macrophages from germfree mice showed a higher basic activity of lysosomal enzymes than did macrophages from conventional mice, whereas oil-induced peritoneal influx, induction of lysosomal enzymes, and phagocytosis via the C3b receptor after endotoxin stimulation were reduced or absent. After germfree mice had been housed with conventional mice for 1 week, peritoneal influx and C3b receptor-mediated phagocytosis reached normal levels; after 4 weeks, enzyme activities also reached normal levels.

Germfree (GF) animals have been widely utilized as models for studies on immunological systems (11, 21, 23, 24), and it has been found that GF animals have altered macrophage functions (3, 4, 6, 8, 9, 13, 22).

Previously, we described differences in enzyme activities and decreases in peritoneal influx, spreading ability, and C3b receptor-mediated phagocytosis in peritoneal macrophages from GF mice as compared with those from conventional (CONV) mice (16). Employing the same techniques, we now report on whether the establishment of a gastrointestinal microflora can influence the above-mentioned properties of peritoneal macrophages.

As before, mice of the NMRI strain, each weighing about 20 g, were used. The GF mice were raised and reared as described previously (7). "Ex-germfree" (EXG) mice were "conventionalized" by taking GF mice out of the isolators (some at 1 and some at 4 weeks before *in vivo* stimulation of macrophages) and placing them in cages with CONV mice. The EXG mice remained healthy throughout the period following conventionalization.

There were no differences in resident peritoneal cell counts in CONV, GF and EXG mice that had been conventionalized for 1 and 4 weeks (Table 1). Intraperitoneal injection of mineral oil caused an increase in the total peritoneal cell number in CONV and EXG mice, and about 75% of the cells showed macrophage characteristics (Table 1). Oil injections did not affect the peritoneal count in GF mice (Table 1). Very few polymorphonuclear granulocytes were observed in any of the exudates by morphological evaluation of stained cells.

Table 2 shows the specific activities of the two lysosomal enzymes tested in lysates of macrophages from CONV, GF, and EXG mice after 6 h *in vitro*. β -Glucuronidase and acid phosphatase activities, the latter on the borderline of statistical significance, were higher in lysates obtained from macrophages from GF mice and EXG mice conventionalized for 1 week than in those from macrophages from CONV mice or EXG mice conventionalized for 4 weeks (Table 2).

In vivo stimulation with endotoxin caused marked rises in acid phosphatase as well as β -glucuronidase activities in macrophages from CONV and EXG mice conventionalized for 4 weeks (Fig. 1). There was a small rise in acid phosphatase

activity in macrophages from GF mice under the same conditions (Fig. 1A), whereas no enzyme increase was obtained in macrophages from EXG mice conventionalized for 1 week (Fig. 1A); in contrast, a decrease was found in β -glucuronidase activity after endotoxin stimulation in the latter mice (Fig. 1B).

The amount of opsonized sheep erythrocytes attached or internalized by means of Fc and C3b receptors is shown in Tables 3 and 4. At 6 h, about 50% of the resident cells from all mice showed both attachment and internalization via the Fc receptor (Table 3). Endotoxin stimulation *in vivo* increased the percentage of Fc-attaching and phagocytosing macrophages (Table 3), whereas oil stimulation caused more efficient attachment but no change in phagocytosis via Fc receptors as compared with resident cells. There were no differences among macrophages from CONV, GF, and EXG animals.

The percentage of resident cells that attached particles by the C3b receptor was somewhat higher (about 70%) than that of cells that attached particles by the Fc receptor, but less than 10% of the resident cells from any type of mice showed internalization of C3b-opsonized sheep erythrocytes (Table 4). Endotoxin or oil stimulation *in vivo* did not increase the percentage of C3b receptor-attaching cells; in fact, oil stimulation even caused a small decrease (Table 4). Internalization of C3b-opsonized particles was, however, promoted by *in vivo* stimulation, and endotoxin had a much stronger effect

TABLE 1. Total cell number of peritoneal exudates^a from CONV, GF, and EXG mice without or after oil stimulation *in vivo*

In vivo stimulus	Mean \pm SE no. of peritoneal cells per mouse ($\times 10^6$) ^b			
	CONV	GF	EXG (1 wk)	EXG (4 wk)
None (resident cells)	5.4 \pm 0.7	5.1 \pm 1.0	6.5 \pm 0.9	6.7 \pm 1.3
Oil	16.6 \pm 4.2 ^c	5.9 \pm 1.2	18.8 \pm 6.2 ^c	27.2 \pm 12.7 ^c

^a Cells in freshly harvested exudates (5) were stained with methyl violet, and the morphology and cell count were evaluated by direct study in a Bürcker hemacytometer.

^b Results are the means of five (EXG mice) to seven (CONV and GF mice) experiments \pm standard errors.

^c The results were subjected to the Wilcoxon paired-comparison test. α -Values below 0.05 were considered to indicate statistically significant differences from nonstimulated mice.

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TABLE 2. Lysosomal enzyme activities^a in macrophages from CONV, GF, and EXG mice

Macrophage type	Mean ± SE enzyme activity (mU/mg of protein) ^b	
	Acid phosphatase	β-Glucuronidase
CONV	5.91 ± 0.85	3.08 ± 0.61
GF	6.50 ± 0.93	4.02 ± 0.82 ^c
EXG (1 wk)	8.27 ± 2.20 ^c	4.62 ± 1.21 ^c
EXG (4 wk)	4.97 ± 0.30	2.11 ± 0.61

^a Assayed at 6 h in vitro (1, 12).

^b Results are the means of five to eight experiments ± standard errors.

^c α < 0.05 compared with macrophages from CONV mice.

than did oil (Table 4). About 70% of macrophages from endotoxin-treated CONV or EXG mice ingested particles by the C3b receptor, whereas only 30% of corresponding macrophages from GF mice contained particles (Table 4). After oil treatment, C3b-mediated phagocytosis increased slightly relative to the resident cells (from <10 to 20%), and there were no differences noted among macrophages from CONV, GF, and EXG mice (Table 4).

In the present study, we established that peritoneal macrophages from GF mice responded much less efficiently to C3b phagocytic stimulation than did corresponding macrophages from CONV mice. The results of the enzymatic studies may indicate that enzyme responses to stimulation are related to the actual basic levels of the enzymes (15), since cells from GF mice expressed higher baseline levels, or they may point to a reduced responsiveness to bacterial endotoxin in macrophages from GF mice. The latter view is strengthened by the feeble triggering of C3b-mediated phagocytosis in these cells by endotoxin stimulation in vivo.

We also found that the reduced response of phagocytosis and enzyme induction to bacterial endotoxin, as well as the influx of monocytes into the peritoneum caused by mineral oil, were enhanced after conventionalization. The elevated basic levels of lysosomal enzymes in macrophages from GF mice were lowered to normal levels in macrophages from EXG mice, suggesting that the intestinal microflora might have an influence upon very different functions in peritoneal macrophages. There were, however, time-dependent variations in the normalization of deficient functions in GF mice. The stimulated peritoneal influx and phagocytosis via C3b receptors in macrophages from GF mice were similar to those in macrophages from CONV mice after only 1 week of conventionalization, whereas leveling of the differences in enzyme activities appeared later in the conventionalization period. These findings may have a bearing on reported age-dependent variations in macrophage functions, which may

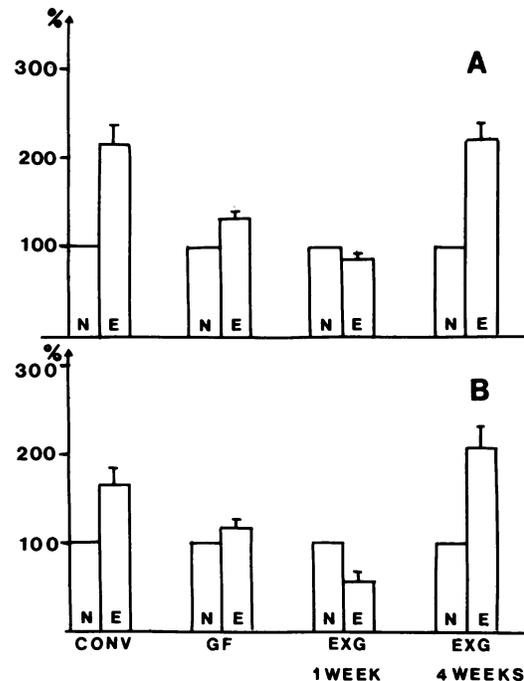


FIG. 1. Effect of in vivo stimulation with endotoxin (E) on lysosomal enzyme activities in macrophages from CONV, GF, and EXG mice. Enzyme activities are calculated as milliunits per milligram of protein (1, 12) and expressed as percentage of corresponding activities in nonstimulated cells (N). Results are the means ± standard errors of the mean of three to five separate experiments. (A) Acid phosphatase; (B) β-glucuronidase.

influence the pathogenesis of bacterial infections (20) as well as malignancy (26) in neonates. Studies on macrophage differentiation in vitro have also indicated time-dependent variations in expression of different functions (17).

Four weeks of conventionalization of GF mice not only caused a reversal of the defects in macrophage functions but seemed to render these functions more activated than those observed in CONV mice (Tables 1, 2, and 4). This finding is in accordance with others (4).

Conventionalization of GF animals has been used to study several aspects of the immune response, such as delayed type hypersensitivity (25), resistance to infections (8, 10), degradation of antigens (3) and enzyme activities (25) in macrophages, and natural killer cell activity (2). In agreement with the present study, all of these reports conclude that immune mechanisms can be modulated by the acquisition of intestinal flora. In accordance with this statement, Pabst et al. (18) have proposed that human monocytes

TABLE 3. Phagocytosis mediated by Fc receptors in macrophages from CONV, GF, and EXG mice without or after in vivo stimulation^a

In vivo stimulus	Mean ± SE % of macrophages from following mice ^b :							
	CONV		GF		EXG (1 wk)		EXG (4 wk)	
	Attached	Internalized	Attached	Internalized	Attached	Internalized	Attached	Internalized
None	51 ± 13	33 ± 11	56 ± 10	34 ± 10	62 ± 20	44 ± 13	63 ± 21	47 ± 16
Endotoxin	77 ± 13	65 ± 16	80 ± 3	63 ± 16	90 ± 6	75 ± 4	89 ± 5	77 ± 5
Oil	76 ± 6	44 ± 16	78 ± 4	35 ± 6	81 ± 8	33 ± 13	73 ± 6	27 ± 7

^a Cells were harvested 3 days after intraperitoneal injection of 70 μg of endotoxin or 1 ml of mineral oil. Opsonized (immunoglobulin G) sheep erythrocytes (0.25 ml of 1%) were added to 0.4 × 10⁶ macrophages for 30 min at 4°C for attachment and 60 min at 37°C for internalization (14). The results are expressed as the percentage of cells that attached or internalized particles, calculated by counting 500 cells per culture under a phase microscope, and are the means of four (EXG mice) to six (CONV and GF mice) experiments ± standard errors.

TABLE 4. Phagocytosis mediated by C3b receptors in macrophages from CONV, GF, and EXG mice without or after in vivo stimulation^a

In vivo stimulus	Mean \pm SE % of macrophages from following mice ^b :							
	CONV		GF		EXG (1 wk)		EXG (4 wk)	
	Attached	Internalized	Attached	Internalized	Attached	Internalized	Attached	Internalized
None	67 \pm 15	9 \pm 5	73 \pm 10	9 \pm 2	73 \pm 12	13 \pm 9	72 \pm 11	9 \pm 4
Endotoxin	82 \pm 7	66 \pm 10	76 \pm 8	28 \pm 6 ^c	75 \pm 17	61 \pm 22	71 \pm 16	74 \pm 12
Oil	59 \pm 11	21 \pm 5	55 \pm 3	19 \pm 5	58 \pm 19	22 \pm 6	56 \pm 18	17 \pm 1

^a Sheep erythrocytes were opsonized with immunoglobulin M plus complement factors in C5-deficient mouse serum (14). Attachment and internalization were assayed as described in Table 3, footnote a.

^b Results are the means of five separate experiments \pm standard errors.

^c $\alpha < 0.05$ compared with macrophages from CONV mice.

require exposure to bacterial products, probably absorbed from the gut, to function optimally.

Prior contact with microorganisms is obviously not essential for all macrophage functions. In the present study, Fc receptor-mediated phagocytosis was similar in peritoneal macrophages from CONV, GF, and EXG mice. It is not known whether this difference between Fc and C3b receptor functions might be related to the appearance of these receptors at different times during cell differentiation (19).

Some studies have reported less effect by intestinal colonization of GF animals with few microorganisms (2). Preliminary data from our laboratory indicate that the normalization of macrophage functions obtained by a complex intestinal microflora was not found after contamination of mice with an *Escherichia coli* strain. Further investigation of the control mechanisms of peritoneal macrophage functions are in progress.

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