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Ontogeny of Macrophage Function to Release Superoxide Anion in Conventional and Germfree Mice

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To determine whether the presence of bacterial flora contributes to the ontogenic development of macrophage function, the ability of macrophages to release superoxide anion (O_2^-) in response to stimulation with phorbol myristate acetate was compared in conventional and germfree mice of various ages after birth. One-week-old conventional mice showed a very low level of O_2^- release by their macrophages, and gradual increases were observed in 2-, 3-, and 4-week-old mice in an age-dependent manner. Macrophages from germfree mice always showed a significantly lower level of O_2^- release compared with conventional mice of the same age; however, age-dependent functional development was seen also in the germfree group. The poor level of O_2^- release by macrophages from adult germfree mice could be restored to more than the level by conventional mice when the mice were conventionalized for 3 weeks. These results suggested that the ontogenic development of macrophage function is not controlled by the presence of bacterial flora but that the full-scale expression of function at each age is under the influence of microflora.

Macrophages play an important role in the protection of the host against a variety of bacteria. These cells migrate into the infected site, phagocytize bacteria, and display intracellular bactericidal ability. In addition to their function as phagocytic cells, they are also very important as accessory cells in the induction and regulation of immune responses.

A functional immaturity of cells of the macrophage series has been implied in hosts at a very young stage. Macrophages from neonatal animals are reported to have impaired accessory functions (3, 8, 12) and to be immature in the expression of defense against viruses and bacteria (4, 22). This immaturity of macrophage functions may account for the susceptibility of neonates to various infections.

In a previous study, we investigated the ontogenic development of macrophage functions (14) which are important in the expression of defense against infection by *Listeria monocytogenes* (10). Mice were highly susceptible to *L. monocytogenes* at 1 week after birth and showed a protection maturing in an age-dependent manner in vivo. Chemotaxis and intracellular killing activity of macrophages in vitro were very poor in younger mice and their functional maturation correlated very well with age-dependent development of in vivo protection. It was obvious that macrophages gradually mature in their function to reach the adult level; however, it has not been determined yet what kind of factor(s) contributes to such age-dependent functional maturation. Among several factors which may have an influence on macrophage function is bacterial flora. The fetus is aseptic, but certain bacterial flora resides in neonates quite soon after birth. It is well known that fecal microflora of neonates shows a consistent pattern of appearance and is different from that in adults (1, 16, 17). Therefore, it is plausible that bacterial flora has a role, at least to some extent, in the functional maturation of macrophages in accordance with animal development. In this respect, germfree mice seem to be a useful tool for investigating the possible contribution of bacterial flora to age-dependent macrophage maturation. There have been several reports on the macrophage function of germfree animals (2, 5, 11), but only adult animals were examined.

In the present study, groups of mice of different ages, raised and maintained under conventional or germfree conditions, were prepared. Peritoneal exudate macrophages were examined for ability to release superoxide anion (O_2^-), one of the important reactive oxygen intermediates contributing to bacterial killing, to investigate the role of bacterial flora in age-dependent functional maturation of macrophages.

MATERIALS AND METHODS

Animals. Colony-bred ICR mice were raised and maintained under conventional or germfree conditions in the animal facilities of Yakult Institute. Conventional mice were maintained in a clean air-conditioned animal room and given laboratory chow and water ad libitum. Germfree mice were maintained under germfree conditions in flexible plastic isolators. Cages, bedding, water, and chow for germfree mice were sterilized in an autoclave. They were taken out of the plastic isolator just before sacrifice for an experiment. In one experiment, germfree mice were converted to maintenance under conventional conditions at the age of 7 weeks and were conventionalized for a further 3 weeks. Age-matched conventional and germfree mice were used 1, 2, 3, 4, 8, and 10 weeks after birth.

PEC. To induce macrophage-rich peritoneal exudate cells (PEC), mice were injected intraperitoneally with 0.04 ml of 10% Proteose Peptone (Difco Laboratories, Detroit, Mich.) per g of body weight. Three days after the injection, PEC were collected by injecting ice-cold Hanks balanced salt solution, pooled, and washed repeatedly. Cells were counted and adjusted to the desired concentration. The viability of cells was always >99% by the trypan blue dye exclusion test. Differential cell counts were performed on Giemsa-stained smears under light microscopy.

Measurement of O_2^- release from macrophages. Superoxide anion (O_2^-) released from peritoneal macrophages was

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TABLE 1. O_2^- release by peritoneal macrophages from conventional and germfree mice[a]

Age of mice (wk)[b]	O_2^- (nmol/min) per 5×10^5 cells		P value
	Conventional	Germfree (% of conventional)	
1	0.066 ± 0.030	0.033 ± 0.028 (50.0)	NS[c]
2	0.124 ± 0.025	0.049 ± 0.019 (39.5)	<0.005
3	0.330 ± 0.064	0.163 ± 0.031 (49.4)	<0.005
4	0.375 ± 0.056	0.238 ± 0.064 (63.5)	<0.05
8	0.445 ± 0.096	ND[d]	

[a] Cells were harvested from 10 to 14 1- or 2-week-old mice and 4 to 8 3-, 4-, and 8-week-old mice and pooled for an experiment. Results are the mean ± the standard deviation of three experiments.
[b] Mice were injected intraperitoneally with 10% Proteose Peptone (Difco) at the indicated age, and PEC were harvested 3 days later.
[c] NS, Not significant.
[d] ND, Not done.

measured by the ferricytochrome *c* reduction method, using phorbol myristate acetate (PMA) as a trigger (9). Cells were suspended in Krebs-Ringer phosphate solution with glucose (137 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 5.7 mM Na_2PO_4, 5.5 mM glucose, pH 7.35) at a concentration of 5×10^5 to 10×10^5 cells per ml. Cells were held in a photometric cuvette and preincubated at 37°C for 5 min after addition of 80 μM ferricytochrome *c* (type VI; Sigma Chemical Co., St. Louis, Mo.) and then added with 100 ng of PMA (Sigma) per ml dissolved in dimethyl sulfoxide (Aldrich Chemical Co., Milwaukee, Wis.). The final concentration of dimethyl sulfoxide was 0.033%. The cuvette was placed in a thermostatted cuvette holder at 37°C, and cytochrome *c* reduction was measured with a two-wavelength spectrophotometer (Hitachi 200-20) at 540 to 550 nm. The amount of O_2^- released after triggering with PMA was calculated from the linear reduction of ferricytochrome *c* (change in optical density per minute) by taking the molar extinction coefficient as 19.1×10^3/M. Results were expressed as nanomoles of O_2^- per minute.

Statistics. The statistical significance of the data was determined by Student's *t* test.

RESULTS

Age-dependent maturation of O_2^- release by macrophages from conventional and germfree mice. Groups of conventional or germfree mice were prepared so that mice of various ages were available for the experiment at the same time. Peritoneal macrophages were obtained 3 days after intraperitoneal injection with peptone at each age in weeks after birth. As we reported previously, there was no significant difference in percentage of macrophages in pooled PEC in any age group; whole PEC were used as peritoneal macrophages. Polymorphonuclear cells (PMN) comprised only 3 to 5% of whole PEC in all age groups, and 50 to 55% of PEC were identified as macrophages by morphological criteria. In conventional mice, the amount of O_2^- released by macrophages after stimulation with PMA was very small at 1 week of age, indicating the functional immaturity of macrophages. The amount observed in this age group was only 15% of the 8-week adult level, and it increased in an age-dependent manner (Table 1). Macrophages from 3-week-old mice showed fivefold higher O_2^- release than those from 1-week-old neonates, which was >80% of the adult level. When age-matched germfree mice were examined for O_2^- release by macrophages, it was found that macrophage function was always poorer when compared with that of conventional mice of the same age. O_2^- release in germfree mice was approximately half that in conventional mice at any age in weeks (Table 1).

Comparison of O_2^- release by macrophages from adult mice raised under different conditions. In the above experiment, both groups of mice were of the same ICR strain. However, groups of conventional mice were bred from breeding mothers which had been maintained under conventional conditions for a long period, and the germfree group were from a mother which had been maintained under germfree conditions. It was still doubtful whether the observed difference between conventional and germfree groups was a simple reflection of the presence or absence of bacterial flora. To answer this, newborn germfree mice were divided into three groups. One group was maintained under germfree conditions until the time of the experiment. The second group was conventionalized at the end of the suckling period and maintained under conventional conditions for a further 7 weeks. The last group was under germfree conditions inside plastic isolators until 7 weeks of age and then were conventionalized for 3 weeks, since it is thought that 3 weeks are required for a complete conventionalization. All groups of mice, which were born at almost the same time from the same group of germfree mothers, were used for experiments 10 weeks after birth. The mean number of recoverable PEC was slightly lower in the germfree group, but differential cell analysis showed that the cell components were the same among the three groups (Table 2). About 55% of PEC were macrophages in every group, and the percentage of PMN was very low. Therefore, superoxide release was determined with the same number of whole PEC from each group without further purification. Macrophages from the germfree group released a very small amount of O_2^-, with a statistical significance when compared with those from conventional mice. On the other hand, macrophage function in conventionalized germfree mice was found to be fully matured (Table 3). There was a significant increase in O_2^- release when germfree mice were conventionalized, and actual data were even higher than those of the conventional group, though statistical significance was not obtained. To deter-

TABLE 2. Differential analysis of PEC from mice raised under different conditions

Group of mice[a]	No. of mice	Mean no. of PEC (10^6) per head	% Cells[b]				
			PMN	Mo	Lym	Eo	Baso
Conventional	6	6.0 ± 1.2	6	57	35	1	1
Germfree	5	4.6 ± 0.9[c]	4	54	41	1	0
Germfree to conventional[d]	5	5.6 ± 1.8	5	56	36	0	3

[a] All mice were used at 10 weeks of age.
[b] Differential cell analysis was performed on Giemsa-stained smears of pooled samples by morphological criteria. Mo, monocytes; Lym, lymphocytes; Eo, eosinophils; Baso, basophils.
[c] $P < 0.05$ versus the conventional group.
[d] Germfree mice were raised under germfree conditions for 7 weeks after birth and then were conventionalized for 3 weeks.

TABLE 3. Comparison of O_2^- release by peritoneal macrophages from mice raised under different conditions[a]

Group of mice[b]	O_2^- (nmol/min) per 5×10^5 cells	P value
Conventional	0.403 ± 0.047	<0.05
Germfree	0.225 ± 0.078	<0.05
Germfree to conventional[c]	0.576 ± 0.114	

[a] Cells were harvested 3 days after an intraperitoneal injection of 10% Proteose Peptone from each group consisting of five to six mice and were pooled for the experiment. Results are the mean ± the standard deviation from three experiments.
[b] All mice were used at 10 weeks of age.
[c] Germfree mice were raised under germfree condition for 7 weeks after birth and then were conventionalized for 3 weeks.

mine whether this trend is true with different cell numbers, the cell concentration was changed. The difference among these three groups was always seen in all cell concentrations examined (Fig. 1). Addition of superoxide dismutase (Sigma) at a concentration of 100 ng/ml completely abrogated the response, indicating that this assay is solely measuring O_2^- release. Judging from the cell number-dependent O_2^- release in all groups, it was obvious that the difference in macrophage content within the range of 2 or 3% is negligible in comparing the results from each group.

DISCUSSION

Among various characteristics displayed by macrophages, the release of reactive oxygen intermediates upon stimulation is the most important one, relating directly to intracellular killing of ingested bacteria. This ability is known to be enhanced when cells are incubated with macrophage-activating agents including lipopolysaccharide, muramyl dipeptide (15), and lymphokine (13). BCG-induced activation of fixed liver macrophages, Kupffer cells, resulted in a significant enhancement of O_2^- release upon stimulation with PMA (9). The ability to release O_2^- seems to be a reliable indicator of the functional state of macrophages. Superoxide release by peritoneal macrophages obtained from 1-week-old mice was found to be very poor in this study. The pattern of age-dependent development of this ability was similar to that of chemotactic and bactericidal activity which was reported in our previous study (14). Mice of ≤3 weeks of age are very small in size and the number of recoverable peritoneal macrophages is limited per animal. As the measurement of PMA-triggered O_2^- release was repeated by using pooled PEC, which contained almost the same percentage of macrophages irrespective of the age of the mice, we believe that the observed age-dependent variation can be attributed to the functional variation of each macrophage cell. Van Epps et al. (18) examined PMN taken from various human age groups for their chemiluminescence response to opsonized zymosan and observed a very low response in fetal cord blood PMN and a low response in cells from the group aged 1 to 3 years. The magnitude of the chemiluminescence response of alveolar macrophages from 1-day-old piglets is reported to be lower than that from adult pigs (21). Although the experimental system is different, these results are consistent with the present results.

As one of the candidates for the factor contributing to age-dependent development of macrophage function, the biological significance of bacterial flora was examined by using germfree mice. In every age group, O_2^- release was always lower in germfree mice than in conventional mice.

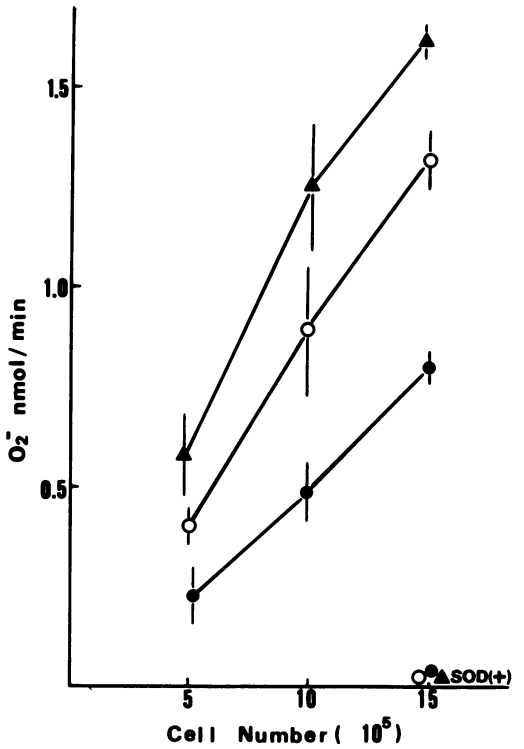

FIG. 1. Release of superoxide anion by peritoneal macrophages in various concentrations in mice maintained under different conditions. Peritoneal macrophages were pooled from groups of five to six conventional mice (○), germfree mice (●), or germfree mice conventionalized for the last 3 weeks (▲). The amount of O_2^- released was measured at cell numbers of 0.5×10^6, 1×10^6, and 1.5×10^6 after stimulation with PMA. Each point and bar indicates the mean ± the standard deviation from three experiments. The symbols in the right lower corner are the results in the presence of superoxide dismutase (SOD).

However, macrophages from germfree mice showed again a functional development in an age-dependent manner of their own.

This result suggested that age-dependent variation is not controlled by the presense of bacterial flora and that there may be some other controlling mechanism. The presence of bacterial flora seemed to have a role in promoting macrophages to display a full-scale function at each developmental stage. This idea can be supported by the fact that an impaired O_2^- release in germfree adults was normalized after 3 weeks of conventionalization. It is not clear whether macrophage functions from every aspect are not fully matured or expressed in germfree mice. Many reports indicated a poor expression of some macrophage functions in germfree animals (2, 5, 11), although there are some showing no difference between germfree and conventional mice (7, 20). Bacterial flora consist of various species of bacteria, and gram-negative bacteria especially reside as intestinal microflora. Lipopolysaccharide of gram-negative bacteria has been reported to be capable of enhancing O_2^- release by macrophages (15), and it has been shown that administration of lipopolysaccharide or monoassociation with *Escherichia coli* in germfree mice resulted in normalization of immune responses (6, 19). It may be possible that gram-negative microflora play a role in the full-scale expression of macrophage function.

In conclusion, it can be said that age-dependent develop-

ment of macrophage function is not controlled by the presence of bacterial flora but rather is under the influence of normal flora.

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