Establishment of a Primary Culture Method for Mouse Intestinal Epithelial Cells by Organ Culture of Fetal Small Intestine

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Studies of the physiological functions of intestinal epithelial cells (IECs) have been limited by the difficulty of primary culture of IEC. We established a method for primary culture of mouse IEC by culturing fragments of fetal small intestines pretreated with EDTA. This method reproducibly resulted in the expansion of cytokeratin-positive epithelial cells, and vigorous expansion of the epithelial cells was observed only from intestinal fragments of embryonic days 15-16. These cells expressed alkaline phosphatase activity and major histocompatibility complex (MHC) class II molecules, indicating the mature phenotype of IEC in a small intestine. The cells also presented antigens to CD4⁺ T cells. Furthermore, the cells expressed various cytokines and chemokines, and the expression was enhanced by bacterial stimulation. These results indicate that the primary-cultured mouse IEC prepared by the method established here can be a beneficial tool in study of the functions of IECs, especially in mucosal immunity.

Key words: intestinal epithelial cells; primary culture; mouse; antigen presentation; innate immunity

Intestinal epithelial cells (IECs) function as a component of the structural and physiological barriers covering the surface of intestinal mucosa, besides functioning as site for digestion and nutrition intake. Most macromolecules, including microbes, are prevented from passing through the tight junctions between IEC under normal conditions.1,2) Food allergens are digested into peptides and further into amino acids by brush border-associated aminopeptidases in IECs, resulting in a decline in allergenicity.3,4) It has been reported that IECs are one of critical components of the gut immune system, with the ability to secrete various cytokines and chemokines,1,5,6) to take up and transport undigested macromolecules,3,7) and to process and present exogenous antigens to T cells.7) IECs also express the polymeric immunoglobulin receptor (pIgR), which transports multimeric IgA and IgM from the lamina propria to the lumen.8) Defensins, one class of antimicrobial peptides, are secreted from IECs.9,10) Expression of major histocompatibility complex (MHC) class II molecules, which are required for antigen presentation to CD4⁺ T cells, has been found in normal IECs of the small intestine, whereas colonic IECs express only low levels of surface MHC class II molecules.7,11)

Proliferation and differentiation of IECs and cytokine secretion from IEC have been reported to be modified by stimulation with commensal and ingested microorganisms in the intestinal lumen.9,10,12) IECs recognize a broad range of microbial components by the mechanisms utilized in the innate immune system, in which conserved pathogen-associated molecular patterns are recognized by pattern-recognition receptors, including Toll-like receptors (TLRs).13) IECs, as well as macrophages and dendritic cells, have been reported to express several cytokines and chemokines in response to stimulation with microorganisms through TLRs.14-18)

In general, primary-cultured cells are expected to retain most of the physiological features of the original cells. For IECs, however, due to difficulties in primary culture with intact physiological functions, studies of the immunological functions of IEC have been very limited. Thus most findings that IECs express molecules involved in immune functions have been obtained from immunohistochemical studies of intestinal sections19,20) or using tumor-derived or oncogene-introduced cell lines. Although a few cell lines have been established spontaneously from primary culture of IEC,21) most mouse IEC lines have been immortalized by introducing the simian virus 40 large T antigen gene, an exogenous oncogene.22-24) It has been found that there are functional differences between primary-cultured and immortalized IECs. For instance, primary IECs purified from the small intestine of adult mice25) expressed MHC class II molecules and presented a soluble antigen to specific T cells without induction by interferon (IFN)-γ, but a mouse IEC cell line, MODE-K did not.22) Thus, it remains critically important to study the immunological functions of IECs using a primary culture.
The mouse is a useful animal model for immunological study, and many findings in immunology have been accumulated using this species. One of the advantages of the use of the mouse is that many inbred mouse strains are available, which make it easy to match the haplotype between T cells and antigen presenting cells (APC). Various genetically modified animals, such as knockout and transgenic mice to assess the importance of molecule of interest in immune responses are also available.

In the present study, we established a new method for the primary culture of mouse IECs by organ culture of the fetal small intestine. We further investigated the ability of the primary-cultured IECs to present an antigenic peptide to specific CD4+ T cells, and the expression of cytokines and chemokines in response to bacterial stimulation.

Materials and Methods

Mice. Female BALB/c mice, 6–8 weeks of age, were purchased from CLEA Japan (Tokyo). Pregnant BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). DO11.10 T-cell receptor (TCR) transgenic mice were backcrossed to BALB/c mice. CD4+ T cells from DO11.10 TCR transgenic mice expressing the transgene-derived TCR specific for a chicken ovalbumin peptide, OVA323-339, in the context of I-A^d. All the mice were maintained in the animal facility at the University of Tokyo and were used in accordance with the guidelines of the University of Tokyo. In all the experiments, adult mice and fetuses were sacrificed by cervical dislocation and decapitation respectively.

Preparation of fetal mouse intestinal fragments. BALB/c fetuses were removed on embryonic days 13-18 (E13-18) by cesarean section from BALB/c mouse (CLEA Japan, Tokyo). Pregnant BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). DO11.10 T-cell receptor (TCR) transgenic mice expressing the transgene-derived TCR specific for a chicken ovalbumin peptide, OVA323-339, in the context of I-A^d. All the mice were backcrossed to BALB/c mice. CD4+ T cells from DO11.10 TCR transgenic mice expressing the transgene-derived TCR specific for a chicken ovalbumin peptide, OVA323-339, in the context of I-A^d. All the mice were maintained in the animal facility at the University of Tokyo and were used in accordance with the guidelines of the University of Tokyo. In all the experiments, adult mice and fetuses were sacrificed by cervical dislocation and decapitation respectively.

Primary culture of mouse intestinal fragments. Ten fragments of mouse fetal small intestine prepared as above were cultured in a Matrigel type I-C (Nitta Gelatin, Osaka) before use. Detection of cultured intestinal epithelial cells (IECs) grown out from intestinal fragments was performed by backcrossing to BALB/c mice. The glass vessels were coated with Cell 0.25 M NaCl, pH 7.5. After incubation for 15 min on ice, the tube was shaken by hand, and the supernatant was replaced with fresh EDTA treatment, the intestines were cut into 1-mm-long fragments and transferred into a tube containing EDTA solution (4.5 mM EDTA, 0.25 M NaCl, pH 7.5). After incubation for 15 min on ice, the tube was shaken by hand, and the supernatant was replaced with fresh EDTA solution. This step was repeated 5 times. For treatment with MatriSperse (BD Biosciences, San Jose, CA, USA), a nonenzymatic extracellular matrix depolymerization reagent, the intestines were incubated with MatriSperse reagent on a 100-mm plastic dish at 4 °C for 4 to 6 h without shaking and then cut into 1-mm-long fragments.

Enzyme histochemistry. The isolated IECs were placed on a glass cover slip or a glass slide attached to plastic chambers and further cultured for 2 days before fixation. The glass vessels were coated with Cell Matrix type I-C (Nitta Gelatin, Osaka) before use. Detection of alkaline phosphatase (AP) activity was performed using a modified simultaneous azo-coupling method, as described previously. Briefly, IECs cultured on glass slides were washed with phosphate-buffered saline (PBS), fixed in acetone at 4 °C for 5 min, and air-dried. After they were washed with PBS, the slides were incubated in a humidified chamber for 10 min at 37 °C with 0.2 M Tris–HCl (pH 8.9) containing hexazotized New fuchsin (0.8 mM as New fuchsin; Merck KGaA, Darmstadt, Germany) and 1 mM naphthol AS-Bi phosphate (Sigma). The slides were washed, fixed with 4% paraformaldehyde (PFA) in PBS, and counterstained with hematoxylin.

Immunohistochemistry. For immunohistochemical staining of cytokeratins, the cells on the slides were fixed with acetone at −20 °C for 10 min and washed twice in PBS. For mouse I-A^d/I-E molecules, the cells were treated with 2 μg/ml of recombinant mouse IFN-γ (Genzyme/Techne, Cambridge, MA, USA) for 24 h, and then fixed with 4% PFA for 10 min at room temperature (RT). The cells were permeabilized and blocked using a blocking solution (PBS containing 0.1% Triton X-100 and 5% normal goat serum) at RT for 1 h. They were subsequently incubated at RT for 2 h with rabbit anti-cytokeratin polyclonal antibodies (DAKO, Glostrup, Denmark; diluted 1:800) or rat anti-mouse I-A^d/I-E monoclonal antibodies (MS/5/14.15.2, BD PharMingen, San Diego, CA, USA) diluted in the blocking solution. After washing with 0.1% Triton X-100 in PBS every 5 min for 15 min, the cells were incubated at RT for 2 h with FITC-conjugated goat anti-rabbit IgG antibodies (Chemicon, Temecula, CA, USA) or FITC-conjugated goat F(ab’)2 fragment anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), followed by washing with PBS. Then the slides were coverslipped and sealed before microscopy. The samples were analyzed with a confocal laser scanning microscope (Fluoview FV500, Olympus, Tokyo) or a fluorescent photomicroscope (AX80, Olympus).

Preparation of primed OVA-specific CD4+ T cells. Spleens were removed from DO11.10 TCR-transgenic mice and crushed mechanically in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo) supplemented with 10% FCS, 0.2% NaHCO3, 2 mM-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 50 μg/ml-mercaptoethanol. The cell suspension was passed through the nylon mesh and centrifuged. CD4+ T cells were purified by positive selection using anti-mouse CD4 MicroBeads, MACS LS Separation Columns, and MACS Separator MidiMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Splenocytes derived from BALB/c mice as APC were treated with 50 μg/ml of mitomycin C (MMC) (Sigma) at 37 °C for 45 min, followed by washing with the medium 3 times. The purified CD4+ T cells (1 × 10^6/well) were cultured in a 24-well plate with APC (1 × 10^7/well) and 0.05 μM OVA232-339 in a total volume of 2 ml. After culturing for 3 d, the cells were harvested, washed, and cultured in RPMI medium containing 10% FCS in the absence of OVA232-339 in a 24-well plate for 4 d.

Antigen presentation to OVA-specific T cells. For assay of antigen presentation to CD4+ T cells, primary-cultured IECs were subjected directly to MMC treatment. The primed CD4+ T cells (5 × 10^5 cells/well) were cultured in 96-well U-bottom plates with MMC-treated IECs (2 × 10^5 cells/well) in the presence and the absence of 0.5 μM OVA232-339 for 96 h. Proliferation was assessed by measuring the incorporation of [3H]thymidine (37 kBq/well) during the final 24 h of culture.

Quantitative RT-PCR. The primary-cultured IECs (4 × 10^5 cells/well) were seeded in a 48-well plate. On the next day, the cells were treated with 1 μg/ml of lipopolysaccharide (LPS) derived from Escherichia coli 0111:B4 (Sigma), or with 30 μg/ml of heat-inactivated E. coli (JCM16495) for 5 h. Isolation of total RNA from the cells, reverse transcription, and quantification of specific cDNA were performed, as described previously, using an Real-time RT-PCR system (Agilent, Hilden, Germany), a QuantTect SYBR Green PCR Kit (Qiagen), and a Light Cycler system (Roche, Mannheim, Germany). The pairs of primers used were as follows: HPRT sense, 5'-CGTGGTAAAAGGGACCTTCTCG-3'; HPRT antisense, 5'-TGAGTTACTTATTTAAGCGGGCA-3'; IL-1β sense, 5'-GGCCCTCAGAAGGGAATGAC-3'; IL-1β antisense, 5'-TGCTGCTGACCTGTTGTTT-3'; TNF sense, 5'-AAATCTGACCTCTCCTCTCG-3'; TNF antisense, 5'-GCTGGTAGAAGTAGATGAG-3'; IL-6 sense, 5'-TGAGTGCAGCAGAGGAGTGATCAGG-3'; IL-6 antisense, 5'-TCTGGATACGGTGGAGAAGTGTCAGCAAG-3'; MIP-1α sense, 5'-AAAGCCTGGATACGGTGTC-3'; MIP-1α antisense, 5'-TGCAGGACACATGTGGGAAC-3'; TGF-β sense, 5'-AGCGCCCTGATACCCG-3'; TGF-β antisense, 5'-AGAGCCTGGATACCCG-3'; MIP-1α (CCL3) sense, 5'-CTCCCGAG-
Expression of intestinal epithelial cell markers by primary-cultured fetal IECs

Next we examined primary-cultured cells for the phenotype of IECs. First, indirect immunofluorescent staining using anti-cytokeratins polyclonal antibodies was performed in order to confirm the intestinal epithelial origins of these cells. Fetal IECs at day 14 of primary culture were stained and observed by confocal laser scanning microscopy (Fig. 1C). Cytokeratins were clearly detected in the cytoplasm of the IECs as reticular fibers, indicating the epithelial nature of these cells.

To identify the phenotypes of the primary-cultured IEC, the AP activity and expression of MHC class II molecules were examined by enzyme histochemistry and immunohistochemistry respectively. AP activity has been found in the brush border of epithelial cells of the small intestine. Most of the cells showed positive staining for AP activity at the cell surfaces (Fig. 1D). It was found that the IECs were clearly stained with anti-mouse MHC class II molecules antibodies even in the absence of IFN-γ stimulation (Fig. 2). Expression of MHC class II molecules in these IECs was enhanced in the presence of IFN-γ, which has been reported to increase expression of them. These results indicate that primary-cultured IEC prepared from mouse fetal small intestines by this method can possess the matured phenotype of IECs.

Responses of primary-cultured fetal IECs to LPS

Finally, we examined the responses of primary-cultured fetal mouse IECs to LPS and to E. coli as a representative gram-negative bacterium. Both stimulation with LPS and with heat-inactivated E. coli increased mRNA expression of cytokines, such as IL-1β, TNF-α, IL-6, and IL-7, in the IECs (Fig. 4A). Moreover, the treatment with LPS and with E. coli increased chemokines known to be expressed in IECs, including MIP-1α (CCL3) and MCP-1 (CCL2). IL-6 and MCP-1 were also detected in the culture supernatant of the IECs without any stimulation (Fig. 4B). When the IECs were stimulated with LPS, secretion of IL-6 and MCP-1 was also enhanced. TGF-β was also detected in the supernatant of the unstimulated IECs. However, neither LPS nor E. coli significantly enhanced mRNA expression or secretion of TGF-β mRNA expression (Fig. 4A) and secretion (data not shown) of thymus-expressed chemokine (TECK/CCL25) were also detected constitutively, but were not changed by the stimulation. These results indicate that primary-cultured fetal mouse IECs have the ability to secrete various cytokines and chemokines and to respond to bacterial components, leading to enhanced secretion.

Discussion

In the present study, we found that primary-cultured cells displaying the functional properties of IECs can be
reproducibly prepared by organ culture of fragments of the fetal mouse small intestine treated with EDTA or Matrisperse. We also found that primary-cultured mouse IECs prepared by this method retained antigen presentation to specific CD4\(^+\) T cells, the production of cytokines and chemokines, and their responses to LPS. Thus the IECs obtained by this new method can be useful in studying the immunological functions of IECs.

In most previous studies that succeeded in primary culture of IECs, the cells isolated from the intestines by vigorous shaking or using dissociating solutions containing EDTA and/or proteases were cultured.\(^{21-23,27,33} \) Following these reports, we tried to culture IECs isolated from the intestines of adult mice, including young germ-free mice, but we were unsuccessful, since no adhesion to the culture dish and no proliferation of isolated IECs was observed reproducibly (data not shown). By culturing fetal intestinal fragments instead of isolated cells after EDTA/Matrisperse treatment, we finally succeeded in the primary culture of mouse IECs (Fig. 5). Fragmentation and shaking of intestinal tissues should be considered critical steps in obtaining epithelial cells. The intestine should be finely cut into pieces rather than mashed. In the step of EDTA-treatment, overly strong shaking should be avoided so that the epithelial layer is not completely detached from the tissue.

We did not observe any expansion of IECs from intestinal fragments of E14 and E17 fetal and postnatal mice except for one example, with E17 mice (Table 1). The fetal intestine in the early developmental stage has a simple structure. In the E14 fetuses, only stratified epithelium was observed, and no obvious villus structure was formed. At E15–E16, the structure of the villi develops rapidly and the epithelium changes from a stratified to a monolayer shape.\(^{34,35} \) Moreover, the structural development of the microvilli and the appearance of brush border enzyme activities occur after E16 in mice.\(^{36} \) Thus at E16 in fetal mouse development the intestinal epithelium might be abundant in dividing cells with potency to differentiate into mature IECs. On the other hand, the developed epithelium after E17 might contain a lower percentage of dividing cells to total...
epithelial cells. The drastic developmental change in the small intestine might be related to our results.

We found that IECs from the fetal mouse small intestine cultured in vitro for 2 weeks had the ability to present exogenous antigens to specific CD4⁺ T cells. It has been reported that mature mouse IECs freshly isolated from adult small intestines can process exogenous antigens and present them to antigen-specific mouse T-cell hybridomas. 25) Generally, however, the problem arising in experiments using primary IECs isolated from adult mice is a remarkably low level of viability of IECs. In the case of immortalized cell lines transformed with tumor genes, there is a possibility that they might exhibit phenotypes of immature IECs and might not have the ability of antigen presentation. For instance, although SV40 large T antigen has been used to immortalize primary IECs, many of the cell lines obtained have shown a cryptal cell feature.23) MODE-K cells immortalized by SV40 large T antigen have shown neither expression of MHC class II molecules nor activation of antigen-specific T cell hybridomas under normal culture conditions without IFN-γ treatment.22) In contrast, our results suggest that normal fetus-derived primary cultured IECs can express MHC class II molecules and present exogenous antigen to T cells, as found for IECs isolated from the adult mouse intestine.25)

IECs differentiate from immature crypt cells into mature absorptive enterocytes during migration toward the tip of the villi. Alkaline phosphatase (AP) is an enzyme on the brush border membrane of mature enterocytes. It is considered to be one of the differ-

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**Fig. 4.** Expression of Cytokines and Chemokines by IECs in Response to LPS and Escherichia coli.

A, Primary-cultured IECs at day 14 were cultured with LPS (1 μg/ml, solid bars) or heat-inactivated E. coli (hatched bars) for 5 h, and then total RNA were extracted. Quantitative RT-PCR analysis was conducted, as described in “Materials and Methods.” Similar results were obtained in three independent experiments. B, After LPS-stimulation for 5 h, the cells were washed and cultured with fresh LPS-free medium for 24 h. The cytokines and chemokines secreted into the supernatants were measured by ELISA. The results are expressed as means of triplicate cultures ± SD. *p < 0.05 compared to the control cultures. Similar results were obtained in three independent experiments.

**Fig. 5.** Scheme of Primary Culture Method for Mouse IECs.

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**Table 1.** Frequency of Appearance of Epithelial-Like Colonies from Small Intestinal Tissues Treated with EDTA and with MatriSperse

<table>
<thead>
<tr>
<th>Embryonic day</th>
<th>Treatment</th>
<th>Number of cultured fragments (A)</th>
<th>Number of fragments attached to dishes (B)</th>
<th>Number of epithelial-like colonies (C)</th>
<th>Number of nonepithelial colonies (D)</th>
<th>Percentage of epithelial-like colonies (B/A (%)</th>
<th>Percentage of nonepithelial colonies (B/A (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>E14</td>
<td>EDTA</td>
<td>98</td>
<td>66</td>
<td>0</td>
<td>66</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>E15</td>
<td>EDTA</td>
<td>2,390</td>
<td>1,970</td>
<td>745</td>
<td>1,225</td>
<td>31.2</td>
<td></td>
</tr>
<tr>
<td>E16</td>
<td>EDTA</td>
<td>1,270</td>
<td>1,143</td>
<td>841</td>
<td>296</td>
<td>66.2</td>
<td></td>
</tr>
<tr>
<td>E17</td>
<td>EDTA</td>
<td>49</td>
<td>39</td>
<td>1</td>
<td>38</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Adult (6–8 weeks)</td>
<td>EDTA</td>
<td>176</td>
<td>81</td>
<td>0</td>
<td>81</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>E15</td>
<td>MatriSperse</td>
<td>2,598</td>
<td>1,793</td>
<td>502</td>
<td>1,292</td>
<td>19.3</td>
<td></td>
</tr>
<tr>
<td>E16</td>
<td>MatriSperse</td>
<td>120</td>
<td>89</td>
<td>33</td>
<td>56</td>
<td>27.5</td>
<td></td>
</tr>
</tbody>
</table>
entiation markers for mature enterocytes. MHC class II molecules are strongly expressed in epithelial cells, located in the two-thirds of the villi from the tip in the small intestine, and are not found in crypt epithelial cells. Therefore, the expression of MHC class II molecules can be considered a marker for mature enterocytes as well. Our finding that primary-cultured IECs had AP activity together with the expression of MHC class II molecules strongly suggest that these cells, in spite of a fetal origin, differentiated to mature enterocytes to a certain extent, and were the same as mature enterocytes, at least in terms of the expression of MHC class II molecules.

Though most of the cells showed positive staining for AP activity (Fig. 1D), the intensity of the staining appeared to be heterogeneous. This might have been caused by the variance in the intensity of AP expression among the IECs, which showed similar levels of maturation, rather than the difference in maturity or the heterogenous cell lineages in the IECs. AP is also used as a biochemical marker to identify enterocytes, mucus-secreting goblet cells, and M cells. Enterocytes express high AP activity, whereas goblet cells and M cells lack it, but it remains to be clarified whether primary-cultured IEC can differentiate into other IEC lineages, goblet cells, enteroendocrine cells, or paneth cells as well as M cells.

No further cell proliferation was observed after harvesting and reseeding of primary-cultured IECs at day 14, though they adhered to the culture vessels (data not shown). Moloney murine leukemia virus (MoMLV) infects only dividing rodent cells. Hence infection by retroviruses can be used as an indicator of cell proliferation. When primary-cultured IECs at day 4 were treated with a retrovirus vector expressing green fluorescence protein (GFP), 20–30% of the cells were found to be GFP-positive (data not shown). These results suggest that the substantial number of proliferating cells contained in primary-cultured IECs might have quit the cell cycle, differentiated, and matured into enterocytes during the 14 d of the culture. This process of differentiation and maturation of primary-cultured IECs might reflect that of proliferating stem cells or precursors in the crypts of a small intestine, which arrest the cell cycle and mature into enterocytes of the villi in vivo.

IECs are known to secrete various cytokines and chemokines. We found that primary-cultured mouse IECs prepared by our procedure expressed cytokines, including IL-7 and IL-6, and chemokines, including TECK/CCL25 and MCP-1/CCL2 (Fig. 4). IL-7 has been reported to enhance IL-4 secretion from CD4+ T cells and to play an important role in the development of intraepithelial γδ T cells, to express TECK/CCL25 is known to be expressed by IECs, and its receptor, CCR9, is mainly expressed by thymocytes and by intraepithelial (IEL) and lamina propria lymphocytes of the small intestine. It is suggested that interaction between TECK and CCR9 plays a role in the homing and migration of IELs into intestinal epithelia and in promoting the induction and function of CD103+ on CD8+ IELs as well as the migration of IgA+ plasma cells into the small intestine. In addition, IL-6 and MCP-1 have been reported to be expressed by IECs. IECs are known to recognize microorganisms through TLRs and to increase the production of cytokines and chemokines. We found that IECs cultured by our method have the ability to respond to the expression of cytokines on recognition of bacterial components. These results suggest that IECs prepared by the procedure described in this report can be used as a useful in vitro model to examine the immunological responses of IECs against microorganisms, including commensal and pathogenic bacteria.

In conclusion, we established a new method for primary culture of IECs from fetal mouse intestines. The cultured cells were found to retain the immunological characteristics of IECs. This primary culture model of mouse IECs might facilitate studies of the functions of particular genes in IEC utilizing the intestines of transgenic and/or gene-targeting mice. Furthermore, primary-cultured IECs might be a good model to analyze IEC responses to intestinal microbiota colonized after birth, because these IECs can be prepared and cultured in germ-free environments.

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References