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Immunological responses to monoassociated *Bifidobacterium longum* and their relation to prevention of bacterial invasion

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Summary. After monoassociation of *Bifidobacterium longum* with germ-free BALB/c mice (nu/+, heterozygous to nu gene), *B. longum* was recovered (10^2–10^4 viable units per organ) from the mesenteric lymph nodes, liver and kidneys for 2 weeks post intragastric inoculation, but not after 4 weeks and later. Cessation of bacterial translocation was not observed in monoassociated nude (nu/nu) mice. Anti-*B. longum* IgA antibody was detected by ELISA using *B. longum* cell wall antigen in bile from Week 1 and in ileac wall extract from Week 8 post-association in both nu/+ and nu/nu mice. Total IgA levels in bile, ileac wall extract and caecal contents were also elevated in both mice after monoassociation. Cell-mediated immunity measured by the footpad test and macrophage migration inhibition test using *B. longum* protein fraction was detected in nu/+ mice in Week 4 and later, but not in nu/nu mice. Nu/nu mice reconstituted 4 weeks prior to monoassociation with lymphocytes from flora-bearing nu/+ mice developed delayed footpad reactivity and bacterial translocation stopped after 4 weeks. Cell-mediated immunity rather than IgA antibody correlated well with the cessation of translocation.

INTRODUCTION

The barrier system of intestinal mucosa against penetration of indigeneous microorganisms or macromolecules therefrom is still not precisely understood, despite the extensive studies to date by many investigators (reviewed by LeFevre, Hammer & Joel, 1979; Bienenstock & Befus, 1980; Tomasi, 1983). Some indigenous enteric organisms can penetrate the barrier and translocate to the internal organs (Wolochow, Hildebrand & Lamanna, 1966; Van der Waaji, Berghuis-de Vries & Lekkerkerk-van der Wees, 1972), especially when the host is raised in the germ-free (GF) state before association of the organisms (Berg & Garlington, 1979) or if the host is athymic (Owens & Berg, 1980); this phenomenon is frequently referred to as bacterial translocation (Berg & Owens, 1979). We recently found that GF mice monoassociated with *Bifidobacterium longum* did not allow this translocation after a certain time period (4 weeks) had elapsed, and such *B. longum*-monoassociated mice also became refractory for the translocation of *Escherichia coli* when the latter organisms were subsequently administered (Yamazaki *et al.*, 1982). This acquired phenomenon of *B. longum*-monoassociated mice attracted our interest because immunological mechanisms might be involved in the development of the barrier against penetration of indigenous enteric bacteria. Thus, the immune responses of *B. longum*-monoassociated mice to the associated *B. longum* were investigated, and the time-course of the appearance of immune responses was compared to the time of development of the acquired resistance to translocation. When these features of euthymic mice were

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compared with those of athymic ones, a lymphocyte-mediated mechanism appeared to be involved in the prevention of translocation.

MATERIALS AND METHODS

Mice
Both sexes of GF nude (nu/nu) and heterozygous (nu/+) mice of BALB/c background were used at 8–12 weeks of age. Methods of maintenance and checking of the GF state were as described previously (Ueda, Yamazaki & Someya, 1973). Barrier-maintained nu/nu and nu/+ BALB/c mice were used at 8 weeks of age as flora-bearing mice.

Bacteria
B. longum strain BB-536, isolated from healthy infant faeces in 1969 by Dr S. Shimamura (Central Institute of Morinaga Co. Ltd) and kept frozen at −76°, was used. The culture of *B. longum* was thawed and inoculated into fresh GAM broth which was then overlaid with sterilized liquid paraffin, and the broth was incubated at 37° for 16 hr. After two subsequent subcultures, the final culture was appropriately diluted with fresh GAM broth in a tube with a rubber cap, and the tubes were introduced into a vinyl isolator.

Inoculation
Mice were given 0·4 ml of the bacterial suspension per head intragastrically using a catheter. The viable units of the inoculum, $2{\cdot}5 \times 10^7$ per ml, were measured by inoculating a portion of the inoculum suspension onto GAM agar plates and incubating the plates in a GASPACK anaerobic system (BBL, Cockeysville, MD) at 37° for 3 days.

Recovery of the bacterium
After inoculation, two to five mice were removed from the isolators at intervals; these were killed by bleeding from the heart under ether anaesthesia for sampling of serum and tissue fluids as described below, and for measuring the viable number of *B. longum* in the liver, kidneys and mesenteric lymph nodes. After weighing these organs aseptically, a portion of each organ was dissected and 1 : 10 tissue emulsions were prepared in a glass homogenizer. Caecal contents were also weighed. These tissue emulsions or caecal contents were inoculated onto GAM agar plates after appropriate dilution with GAM broth and incubated as described above.

Serum and tissue samples for antibody measurement
Serum obtained from the heart blood was heat-inactivated at 56° for 30 min and diluted at 1:5 in phosphate-buffered saline, pH 7 (PBS). Bile was obtained from the gallbladder and diluted to 1 : 10 with PBS containing 0·05% Tween 20 and 0·02% sodium azide (PBS-TSA). About 7 cm of the distal portion of the small intestine was dissected, opened, and rinsed in three changes of approximately 20 ml each of PBS-TSA. After weighing, a tissue emulsion was prepared with a 10-fold volume of PBS-TSA. After centrifuging the suspension at 2290 ***g*** for 15 min, the supernatant was used as tissue extract of the ileac wall. Caecal contents were weighed, suspended in a 10-fold volume of PBS-TSA and centrifuged 2290 ***g*** for 15 min. These samples were used for the measurement of IgA antibody and IgA levels.

Bacterial preparations
For anti-*B. longum* antibody measurement, a cell wall fraction was prepared from the organisms. GAM broth-grown cells of *B. longum* were washed three times with saline and disrupted by a Sorval-Ribi refrigerated cell fractionator. The suspension obtained was centrifuged at 2000 ***g*** for 30 min and the supernatant was centrifuged at 25,000 ***g*** for 40 min. The sediment was suspended in 0·067 M phosphate buffer (pH 8·0) containing 2 mg/ml of crystaline trypsin, 0·05 mg/ml of Sigma RNase (Sigma, St Louis, MO) and 0·01 mg/ml of Sigma DNase, and incubated at 37° for 3 hr. After incubation, the suspension was centrifuged at 25,000 ***g*** for 40 min and washed five times with distilled water. Then, the sediment was suspended in 0·01 N HCl containing 1 mg/ml of crystaline pepsin, and incubated at 37° for 24 hr. The suspension was centrifuged at 25,000 ***g*** for 40 min and washed five times with distilled water. The resulting sediment was dialysed against distilled water for 48 hr and then lyophilized.

In order to measure cell-mediated immunity, a crude protein fraction (BPF) was prepared according to the method of Collins & Mackaness (1968) with some modification. *B. longum* cells were propagated on GAM agar at 37° for 48 hr anaerobically, and grown organisms were suspended in GAM broth and incubated at 37° for 48 hr aerobically. After centrifugation at 6000 ***g*** for 10 min, the sediment was dissolved in PBS and washed by centrifugation. To the sediment was added 100 times the volume of PBS, and the suspension was incubated at 37° for 7 days with four intermittent shakings per day. Then, the suspension

was centrifuged at 10,000 ***g*** for 30 min and the supernatant was filtered through a 0·2 μm millipore filter membrane. Ammonium sulphate was added to the solution, and precipitates generated between 70 and 100% saturation were collected. The precipitate obtained was dissolved in 0·05 M PBS and applied to Sephadex G25. The first peak of protein fraction monitored at 280 nm was collected and lyophilized.

Measurement of antibody and IgA by ELISA

The ELISA method for antibody was essentially according to Iwai *et al.* (1984). The cell wall fraction of *B. longum* was coated in wells of enzyme immunosorbent assay (EIA) plate (Micro-Test flexible assay plates, Falcon No. 3912, Falcon, Oxnard, CA) at 3 μg per well and stored at 4° until use. The wells were filled with 100 μl of PBS containing 1% bovine serum albumin (PBS-BSA), and plates were incubated at 37° for 60 min. After three washings with PBS-BSA, 30 μl of samples was added to each well and plates were incubated at 37° for 40 min. After washing with PBS-BSA containing 0·05% Tween 20, 25 μl of horseradish peroxidase (HRP)-labelled rabbit anti-mouse IgG (1:2500, H- and L-chain specific, purchased from Cappel, Cochranville, PA) were added to wells for serum samples, while 30 μl of HRP-goat anti-mouse IgA (1:800, α-chain specific, Cappel) were added to wells for serum and other samples, and plates were again incubated at 37° for 45 min. Then, after washing, 100 μl of 5-aminosalicilic acid solution (1 mg/ml, pH 6·0) containing 5% H_2O_2 were added to each well and the plates were left at room temperature for 30 min. The absorbances of the contents of the wells were measured at 450 nm using a Titertek Multiscan (Flow Laboratories Inc., McLean, VA). For negative control samples, sera and tissue extracts from non-treated GF nu/+ and GF nu/nu mice were used, and values of absorbance more than 0·1 higher than the controls were considered positive. In order to measure IgA levels, appropriately diluted goat anti-mouse IgA (1:400, α-chain specific, Cappel) was coated on the wells of EIA plates and processed in the same way as for detecting IgA antibody. For negative controls, saline was used instead of samples. Using IgA-producing hybridoma ascitic fluid (a gift from Dr Nariuchi of the Institute of Medical Science, University of Tokyo) as a positive control sample, dilution of the IgA hybridoma fluid up to 20,000-fold showed a linear absorption curve.

Spleen cell transfer

Spleen cells were obtained from flora-bearing nu/+ mice after mincing and filtering through a 200 mesh stainless steel sieve using minimal essential medium (MEM, Nissui Co., Tokyo) supplemented with 3% fetal calf serum (FCS, Flow Laboratories, Rockville, MD). Glass-adhering cells were removed from the suspension by incubation at 37° for 120 min in a CO_2 incubator. The viable count was obtained using the trypan blue dye exclusion method. The cell suspension was introduced into an isolator, and $2{\cdot}5 \times 10^7$ viable lymphocytes per 0·2 ml were injected i.v. via the tail vein of nu/nu mice.

Footpad reaction

Mice were injected with 10 μg of BPF in 0·04 ml of saline into one footpad and with saline into the other, and 3 and 24 hr later footpad thickness was measured with stainless hardened calipers (Mitsutoyo, Tokyo). Increased thickness of the antigen-induced site in comparison with the control of more than 3·5 u (1 u = 0·1 mm) was considered positive.

Macrophage migration inhibition test

Mice were injected i.p. with 2 ml of thioglycolate broth (Difco Co. Ltd, Detroit, MI), and 4 days later peritoneal exudate cells (PEC) were collected from four mice in each group by washing the peritoneal cavity with 3 ml of MEM. After two washings with MEM, cells were packed into capillaries and centrifuged. The capillaries were cut at the packed cell–fluid interface, and the cell-packed portion was placed in a small petri-dish and incubated with MEM containing 15% FCS with or without BPF (25 μg/ml). Migration distance from the cut end of the capillary to the top margin of the migrated area was measured with a micrometer after 24 hr of incubation at 37°. Percentage migration was obtained by comparing the migration distance with and without BPF.

RESULTS

Detection of viable *B. longum* in organs of mono-associated mice

After intragastric inoculation of *B. longum*, two to five mice from both nu/+ and nu/nu groups were killed at intervals and used for the detection of viable *B. longum* and antibodies.

As shown in Table 1, low counts of *B. longum* (usually 10^2–10^4 organisms per organ) were recovered from the liver, kidneys and mesenteric lymph nodes in most nu/+ and nu/nu animals killed in Weeks 1 and 2. In Week 4 and later, no more *B. longum* was recovered

Table 1. Viable numbers of *B. longum* in internal organs after monoassociation

Mice	Weeks after monoassociation	No. mice	No. of *B. longum* per organ (per g tissue) Liver	Mesenteric lymph nodes	Kidney
nu/+	1	3	4·2±0·5*[2/3]† (3·8±0·4)	2·3±0·2 (3·2±0·3)	2·1±0·4[2/3] (2·7±0·6)
	2	3	3·6±0·5[2/3] (3·2±0·5)	1·8±0·2 (2·6±0·7)	1·9±0·2 (2·4±0·3)
	4	5	Negative (<1·7)	2·4[1/5] (3·5)	ND‡
	8	5	4·0[1/5] (3·7)	0·6[1/5] (1·7)	2·0 (2·6)
	12	5	Negative (<1·7)	Negative (<1·7)	Negative (<1·7)
	18	5	Negative (<1·7)	Negative (<1·7)	Negative (<1·7)
nu/nu	1	3	4·6±0·7[2/3] (4·1±0·7)	2·7±0·5 (3·9±0·7)	2·3±0·4 (2·8±0·6)
	2	3	3·9±0·3 (3·6±0·3)	2·0±0·1 (3·6±0·5)	2·3±0·21 (2·9±0·2)
	4	4	3·8±0·5[3/4] (3·6±0·5)	2·8±0·7 (3·8±0·7)	2·1±0·2 (2·6±0·3)
	6	3	3·8±0·3[2/3] (3·6±0·3)	2·0±0·3 (2·8±0·7)	1·8±0·3 (2·4±0·3)
	12	2	3·4±0·5 (3·1±0·4)	2·1±0·4 (2·8±0·2)	ND

* Results are given as $\log_{10}\pm$SD of positive cases.
Values in square parentheses represent the no. positive/no. tested, when not all cases were positive.
ND, not determined.

from these organs of nu/+ mice except on rare occasions, whereas the organisms were consistently isolated from the organs of nu/nu mice. Both nu/+ and nu/nu mice harboured more than 10^9 *B. longum* per gram of caecal contents throughout the observation period.

Antibody responses and IgA levels

Serum antibody. Serum antibody to *B. longum* was measured by ELISA using anti-mouse IgG or α-chain specific anti-mouse IgA as secondary antibodies. In both nu/+ and nu/nu mice, levels of serum antibody before *B. longum* association were less than 1:5 (serum dilution, measured using anti-mouse IgG), and low titres (1:5–1:20) were detected in Week 1. The levels increased gradually and reached 1:40–1:160 in nu/+ mice and 1:20–1:40 in nu/nu mice in Week 12 (data not shown). In both mice, no IgA antibody was detected at a 1:5 serum dilution.

*Anti-*B. longum *IgA antibody in secretory fluids.* IgA antibody to *B. longum* was measured in bile, ileac wall extracts and caecal contents, and the results are shown in Table 2. Samples of 10-fold diluted extract were expressed as 1:10 dilutions. Anti-*B. longum* IgA was first detected in the bile in Week 1 (1:10–1:40), and moderate titres (1:10–1:160) were maintained in the bile throughout in both nu/+ and nu/nu mice. Low IgA antibody titres also became detectable in the cecal contents and ileac wall extracts in Weeks 2 and 6, respectively, in nu/+ mice, and Weeks 6 and 12, respectively, in nu/nu mice.

IgA levels in secretory fluids. In GF nu/+ mice, IgA levels in the bile and ileac wall before association were relatively high compared to the caecal contents and serum. In GF nu/nu mice, the IgA level in the bile only was higher than in the other samples. After *B. longum* association, as shown in Table 3, IgA levels in all samples of both nu/+ and nu/nu mice increased about

Table 2. Anti-*B. longum* IgA antibody in body fluids and tissues of monoassociated mice

Mice	Samples	Weeks after monoassociation								
		0	1	2	3	4	6	8	12	18
nu/+	Bile	<0	1·1±0*	0	2·33±1·53	1·67±1·15	1·0±0	1·5±0·71	0·5±0·71	2·0
	Ileac wall	<0	<0	<0	<0	<0	≦0	0	0	0·5±0·71
	Caecal contents	<0	<0	0	0	0	0·67±0·58	0	0·5±0·71	0·5±0·71
nu/nu	Bile	<0	1·0±1·41	1·5±2·12	–	1·0±1·41	1·0±1·41	–	1	–
	Ileac wall	<0	<0	<0	–	<0	<0	–	0	–
	Caecal contents	<0	≦0	<0	–	<0	1·00±0	–	0	–

* Average of reciprocal of the highest dilution ± SD (log2, × 10^{-1}) measured by ELISA using anti-mouse IgA as the secondary antibody. Number of mice used was two to three per group (except nu/nu mice at Week 12 in which only one mouse was used).

Table 3. Level of total IgA in body fluids and tissues of monoassociated mice

Mice	Samples	Weeks after monoassociation							Flora-bearing mice
		0	1	2	3	4	6	8	
nu/+	Bile	3·33±1·15*	4·67±1·15	4·33±1·53	4·33±1·15	5·33±2·08	7·00±1·00	7·00±0·00	5·67±0·58
	Ileac wall	2·67±1·15	5·00±0·00	3·67±0·58	5·00±1·00	5·33±0·58	6·00±1·00	6·00±1·41	6·33±0·58
	Caecal contents	≦0	3·67±0·58	3·00±1·00	4·33±0·58	3·00±1·00	2·33±1·53	4·00±0·00	1·33±0·58
	Serum	0·33±0·58	3·67±1·53	3·00±1·00	2·33±1·15	4·33±0·58	4·33±0·58	3·50±0·71	4·33±1·15
nu/nu	Bile	2·50±0·71	4·00±1·41	6·00±0·00	–	4·50±0·71	7·50±0·71	–	6·00±1·41
	Ileac wall	≦0	2·50±0·71	3·00±0·00	–	4·00±1·41	5·00±0·00	–	3·50±0·71
	Caecal contents	<0	1·00±1·41	1·50±0·71	–	3·00±1·41	2·50±0·71	–	1·00±0·00
	Serum	0·50±0·71	3·50±0·71	2·50±0·71	–	4·00±1·41	3·00±0·41	–	2·50±0·71

* Average of reciprocal of the highest dilution ± SD (log2, × 10^{-1}) measured by ELISA using solid-phase anti-mouse IgA and labelled anti-mouse IgA as the secondary antibody.

two- to four-fold in Week 1, and the levels increased progressively, especially in the bile and ileac wall of both mice, to the levels of flora-bearing mice in Week 4 or 6.

Cell-mediated immunity (CMI) to associated *B. longum*

Footpad reaction. The BPF antigen from *B. longum* was injected into the footpad of three to five mice at various times after monoassociation. A group of non-associated GF nu/+ and GF nu/nu mice were also tested prior to the monoassociation as a negative control. Footpad swelling was measured 3 and 24 hr after administration (Table 4). In nu/+ mice, the delayed type footpad reaction was negative in Week 2 but turned positive in Week 4, and the reactivity persisted up to Week 18. Considerable levels of immediate (3 hr) swelling were also seen in these mice. Nu/nu mice did not show delayed footpad swelling at any of the times tested, while at a low level the immediate reaction was seen in Weeks 4 and 6.

Macrophage migration inhibition test. Groups of nu/+ and nu/nu mice were monoassociated with *B. longum* and used for the migration inhibition test using PEC in Weeks 2 and 4 (Table 4). Migration inhibition of the peritoneal exudate cells of nu/+ mice in the presence of BPF antigen was negative in Week 2 but positive in Week 4. No migration inhibition was detected in nu/nu mice in Weeks 2 and 4.

Table 4. Cell-mediated immune responses in *B. longum*-monoassociated mice

Mice	Weeks after monoassociation	No. mice	Footpad swelling* (0·1 mm ± SD) 3 hr	24 hr	Macrophage migration inhibition test† (% migration)
nu/+	0‡	4	2·1 ± 0·9	1·1 ± 0·9	ND§
	2	4	1·3 ± 0·6	0·8 ± 0·4	98·8
	4	4	3·8 ± 1·2	4·6 ± 0·8¶	59·1
	6	4	4·1 ± 1·3	5·2 ± 1·4¶	ND
	8	5	7·4 ± 1·1	6·6 ± 1·5¶	ND
	12	5	7·8 ± 2·2	9·0 ± 3·2¶	ND
	18	5	7·2 ± 2·9	3·8 ± 1·0¶	ND
nu/nu	0	4	2·5 ± 0·6	1·0 ± 0·7	ND
	2	4	1·3 ± 0·5	0·6 ± 0·4	102·4
	4	4	3·4 ± 0·9	0·8 ± 0·7	106·0
	6	3	3·8 ± 0·8	0·7 ± 0·3	ND

* BPF 10 μg/0·04 ml/mouse.
† BPF 25 μg/ml. See Materials and Methods.
‡ Before monoassociation.
§ ND, not determined.
¶ Statistically significant ($P<0·05$ or $P<0·01$) by Student's *t*-test against values of GF mice (Week 0).

Reconstitution of nu/nu mice by lymphocyte transfer

GF nu/nu mice received i.v. transfers of 5×10^6 spleen lymphocytes from flora-bearing nu/+ mice 4 weeks before *B. longum* association. Four weeks after association, these reconstituted mice were examined for the footpad reaction with BPF and then killed for the detection of translocation. Monoassociated non-transferred control nu/nu mice were used as a control. As shown in Table 5, the lymphocyte-reconstituted nu/nu mice developed a delayed footpad reaction and *B. longum* organisms were not recovered from the organs, while in non-reconstituted nudes the delayed footpad reaction was negative and *B. longum* was recovered from the organs.

DISCUSSION

After monoassociation of euthymic nu/+ mice with *B. longum*, the organisms could readily be isolated from internal organs such as the mesenteric lymph nodes, liver and kidneys up to Week 2, although the bacterial

Table 5. Recovery of *B. longum* from organs, and footpad reaction in lymphocyte-transferred *B. longum*-monoassociated nude mice

Lymphocyte transfer	Recovery of *B. longum* from* Liver	Mesenteric lymph nodes	Caecal contents	Footpad swelling† after 24 hr
+	0/4‡ (<2·0)	0/4 (<2·0)	4/4 (9·6 ± 0·2)	3·2 ± 1·5§
–	4/4 (3·0 ± 0·3)	4/4 (3·5 ± 0·6)	4/4 (9·7 ± 0·1)	0·4 ± 0·7

* Four weeks after *B. longum* monoassociation.
‡ Positive/examined (viable counts/organ or gram of caecal content).
† BPF, 10 μg/0·04 ml/mouse, was injected.
§ 0·1 mm ± SD.

counts isolated were usually low. This bacterial translocation ceased at about Week 4. In contrast to nu/+ mice, this prevention of translocation was not observed in nu/nu mice. A similar phenomenon has been observed with *Propionibacterium acnes* (Abe *et al.*, 1981) and in specific pathogen-free nu/nu mice (Owens & Berg, 1980). The requirement of lymphocytes in this preventive phenomenon and in the delayed footpad reaction to *B. longum* antigen was confirmed by an experiment using nu/nu mice reconstituted with lymphocytes from nu/+ mice. Since recovery of the delayed footpad reaction is known to be achieved by transfer with splenic T cells from syngeneic mice (Ueda *et al.*, 1982), the lymphocytes effective in this reconstitution experiment seem to be T lymphocytes. These findings, as well as the need of the time-lapse for the development of resistance to translocation, suggest that immunological mechanisms might be involved in this phenomenon. In contrast to *B. longum* or *P. acnes*, no such acquired phenomenon has been observed when *Escherichia coli* was monoassociated (Maejima & Tajima, 1973; Yamazaki *et al.*, 1982). The reason for this difference due to the kind of microbes is not known at present.

Both nu/+ and nu/nu mice produced anti-*B. longum* IgA antibody, suggesting that antigenic stimulation occurred through the intestinal mucosa. The antibody could be detected in bile in Week 1, and thereafter in both mice. IgA antibody in the ileac wall, which probably represents IgA antibody levels in the lamina propriae, could be detected long after the association: Week 6 in nu/+ and Week 12 in nu/nu mice. These findings seem to indicate that a rapid and intensive biliary transfer of anti-*B. longum* antibody from the blood stream (Vaerman *et al.*, 1982) occurred after monoassociation. IgA antibody was negative in the serum, probably due to their presence at too low an amount to be detectable by the present method. This anti-*B. longum* IgA production seems to be accompanied by an elevation of total IgA levels in these tissue fluids. The reason for the early rise in total IgA levels after monoassociation needs further investigation, since polyclonal B-cell stimulation (Nash *et al.*, 1969; McGhee *et al.*, 1982; Spalding *et al.*, 1984) might occur in the present situation. It is of interest to note that increased total IgA levels were also seen in monoassociated nu/nu mice. This finding suggests thymus independency of IgA production under particular conditions.

The time of appearance of anti-*B. longum* antibody in monoassociated mice does not seem to correspond well to the time of prevention of translocation. In addition, the good IgA response in nu/nu mice, which lacked the ability to prevent translocation, also indicates that the IgA response does not play a major role in the cessation of translocation. The prolonged production of anti-*B. longum* IgA antibody continues long after the cessation of translocation, suggesting that not only live *B. longum* organisms detected in the organs, but also stimulation by intraluminal *B. longum* organisms or their product(s), are responsible for immunogenic stimulation leading to IgA antibody production.

In contrast to the humoral immunity, CMI response to *B. longum* protein antigen was detected at week 4 and later in nu/+ mice, but not in nu/nu mice. The time of the development of CMI to *B. longum* seems to correspond to the time of cessation of translocation. Further evidence supporting the possible involvement of CMI in the acquired preventive phenomenon may be the simultaneous development of both prevention of the translocation and development of delayed hypersensitivity in lymphocyte-reconstituted nu/nu mice. There are only a few reports in the literature on observations of CMI produced by intestinal antigenic stimulation (Fimmel & Keast, 1974; Perrotto *et al.*, 1974; André & André, 1976; Huntley, Newby & Bourne, 1979), except for entero-invasive pathogens (Carter & Collins, 1974; Collins & Carter, 1974; Frederick & Bohl, 1976; Collins, 1979; MacDonald & Carter, 1980). Our findings may provide a model system to explore the biological significance of systemic CMI in indigenous enteric microbes.

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