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Lymphocyte Proliferative Response to Viral Antigen in Pigs Infected with Transmissible Gastroenteritis Virus

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Development and sequence of lymphocytes reactive to viral antigen in Peyer's patches, mesenteric lymph nodes, spleen, and peripheral blood of pigs orally inoculated with transmissible gastroenteritis virus were investigated by a lymphocyte proliferative assay. Lymphocytes reactive to the viral antigen were first detected in all the tissues of pigs tested on postinoculation day 7. Thereafter, they increased in proliferative reactivity and reached a maximal amount on postinoculation days 10 to 14. Antigen-reactive cells were persistently demonstrated in Peyer's patches and mesenteric lymph nodes for at least 110 days after inoculation, although lymphocytes decreased a little in reactivity to the viral antigen with the lapse of time. On the other hand, splenic and peripheral blood cells were found to have only transient proliferative reactivity. No antigen-reactive cells were detected in spleen or peripheral blood after postinoculation days 20 to 30. Lymphocytes decreased remarkably in reactivity to the viral antigen and phytohemagglutinin when treated with anti-porcine thymocyte serum and complement. Their reactivity to lipopolysaccharides was hardly affected by the treatment. Cells harvested on postinoculation days 45 to 60, however, responded a little to the viral antigen even after they were treated with anti-porcine thymocyte serum and complement. Lymphocytes reactive to the viral antigen and phytohemagglutinin belonged mainly to the erythrocyte rosette-forming cell fraction, whereas those reactive to lipopolysaccharides were mostly found in the rosette-nonforming cell fraction.

Although numerous investigations have been made on cell-mediated immunity (CMI) to various viral diseases, only a few reports (4, 18) have been published on CMI to gastrointestinal infection with virus.

Transmissible gastroenteritis (TGE) of pigs is a highly contagious enteric viral disease. A disease characterized by severe diarrhea and vomiting and showing a high fatality among newborn piglets is caused by a coronavirus (13), which infects epithelial cells of the small intestine. Pigs that have recovered from a primary TGE infection usually resist challenge exposure to the virulent virus. No mechanism of this active immunity to TGE is known. No serum antibody provides immunity to pigs for the prevention of reinfection (5, 6). One possible explanation for the mechanism of active immunity is that TGE antibody of the secretory immunoglobulin A class in intestinal secretions may protect the mucous membrane of the small intestine from reinfection (12). According to Kodama et al. (Y. Kodama, M. Ogata, and Y. Shimizu, Proc. 83rd Meet. Japanese Society of Veterinary Science, abstract, p. 14, 1977), however, TGE antibody is seldom detected by neutralization test of intestinal secretions collected from pigs that have recovered from TGE, although it can be demonstrated by radioimmunoassay.

Another mechanism that might be responsible for active immunity is local CMI in the small intestine. Frederick and Bohl (4) found that the production of a macrophage migration-inhibition factor by lymphocytes obtained from the lamina propria of the small intestine was greater than that by splenic lymphocytes in pigs that had been orally exposed to TGE virus, and that the reverse was true for pigs subcutaneously exposed to the virus. The duration of CMI, however, remained obscure, because the number of pigs used in their experiment was not large enough. Woods (18) demonstrated CMI in pigs orally exposed to TGE virus by the direct leukocyte migration-inhibition test. Because he examined only peripheral blood leukocytes collected within 35 days after inoculation, the duration of CMI in infected pigs or the aspect of local CMI in the small intestine was unknown.

In the present experiments, the occurrence of CMI was examined in Peyer's patches, mesenteric lymph nodes, spleen, and peripheral blood of pigs orally inoculated with virulent TGE virus

by a lymphocyte proliferative assay in vitro. Further, attempts were made to determine the subpopulation of cells positive for proliferative response in the assay.

MATERIALS AND METHODS

Virus strains. The virulent Shizuoka strain (9) of TGE virus was used for oral inoculation of pigs. It had been passed 17 times in piglets and stored at −80°C in the form of a 10% suspension of the infected small intestine.

The attenuated TO strain of TGE virus was used for production of antigen to be used for lymphocyte stimulation in vitro. It had been passed serially in pig kidney cell cultures (6). It was at passage 168 when used in the present studies.

Experimental pigs. A total of 32 Yorkshire pigs, 2 to 3 months old, that had been serologically negative for TGE antibody were obtained from a farm where no outbreaks of TGE had been reported. Of these, 27 were inoculated orally with 10^5 50% pig infective doses of virulent TGE virus. The remaining five pigs served as uninfected controls.

TGE viral antigen for lymphocyte stimulation. TGE viral antigen used for lymphocyte stimulation in vitro was prepared from pig kidney cell cultures infected with the attenuated TO strain. Infected cultures were frozen and thawed twice, and the culture fluid was clarified by centrifugation at 1,500 × *g* for 20 min. The harvested fluid, which contained virus at a titer of $10^{6.8}$ 50% tissue culture infective doses per ml, was concentrated by ammonium sulfate precipitation and then treated with fluorocarbon (11). After infectious virus was inactivated by treatment with 0.4% solution of β-propiolactone, the antigen solution was dialyzed against four changes of a large volume of Eagle minimal essential medium (EMEM) and stored at −80°C. An antigen solution was similarly prepared from virus-uninfected pig kidney cell cultures and used as a control.

Nonspecific mitogens. Phytohemagglutinin-P (PHA) and lipopolysaccharides (LPS) derived from *Escherichia coli* type O111:B4 were purchased from Difco Laboratories, Inc. (Detroit, Mich.). Mitogens were dissolved in EMEM and stored at −80°C.

Preparation of cell suspensions. Pigs were killed at various intervals after virus inoculation. Lymphoid cell suspensions were prepared from Peyer's patches, mesenteric lymph nodes, spleen, and peripheral blood of these pigs.

Peyer's patches were scraped off with a scalpel, minced, and suspended in cold EMEM. Then cells were dispersed by a gentle agitation on a magnetic stirrer. Mesenteric lymph nodes and spleen were teased in cold EMEM with needles. All the cell suspensions obtained were passed through an 80-mesh stainless-steel screen and subsequently through a glass wool-packed column. One volume of splenic cell suspension was treated with 3 volumes of 0.83% NH_4Cl-tris(hydroxymethyl)aminomethane solution to lyse contaminating erythrocytes. Peripheral blood lymphocytes were separated from defibrinated blood by centrifugation at 400 × *g* for 30 min on a Ficoll-Conray gradient (24 parts of 9% aqueous solution of Ficoll and 10 parts of 33.4% solution of Conray 400 [sodium 5 acetamide-2,4,6-triiode-*N*-methylisophthalmate]). The resulting lymphocyte-rich middle fraction was collected and diluted 1:2 with EMEM. After centrifugation at 400 × *g* for 10 min, pelleted cells were suspended in EMEM.

All the cells were washed three times with EMEM and finally suspended to a concentration of 4×10^6 viable cells per ml in a culture medium consisting of EMEM supplemented with 20% fetal calf serum, 0.175% sodium bicarbonate, 50 μg of kanamycin per ml, and 50 μg of gentamicin per ml. When determined by a trypan blue dye exclusion test, the viability of cells prepared from Peyer's patches, mesenteric lymph nodes, spleen, and peripheral blood was 83 ± 7, 87 ± 7, 78 ± 9, and 98 ± 2%, respectively, and the suspension of these cells contained 15 ± 5, 55 ± 10, 37 ± 8, and 53 ± 9% erythrocyte rosette (ER)-forming cells, respectively.

Separation of ER-forming cells. Each lymphocyte suspension was mixed with an equal volume of a 0.5% suspension of sheep erythrocytes, incubated at 37°C for 10 min, centrifuged at 200 × *g* for 5 min, and further incubated in an ice bath for 1 h. The cell mixture was gently suspended and centrifuged at 400 × *g* for 30 min on a Ficoll-Conray gradient. The resulting pelleted cells with sheep erythrocytes (an ER-enriched fraction) and those with ER-nonforming interphase cells (an ER-depleted fraction) were collected separately from each other. The former were treated with 0.83% NH_4Cl-tris(hydroxymethyl)-aminomethane solution to lyse sheep erythrocytes, and then washed three times with EMEM.

Detection of cell surface markers. The ER and erythrocyte-antibody-complement rosette formation tests were carried out to detect cell surface markers. The procedures of the ER formation test for T lymphocytes have been previously described (10). A slight modification of the erythrocyte-antibody-complement rosette formation test (10) was used to detect cells possessing receptors for complement. Chicken erythrocytes coated with guinea pig anti-chicken erythrocyte antibody and mouse complement were prepared and used in the test.

Goat ATS. Goat anti-porcine thymocyte serum (ATS) was prepared from goats by multiple injection of porcine thymocytes. One volume of the antiserum was heat inactivated and adsorbed twice with 1 volume each of packed porcine erythrocytes and bone marrow cells. The serum inhibited completely the ER formation of thymocytes in a dilution up to 1:80 and killed more than 90% of the thymocytes in a dilution up to 1:320 in the presence of complement, but it had no effect on bone marrow cells.

Lymphocyte proliferative assay. A slight modification of the methods described by Valentine (16) was used to measure lymphocyte proliferation. Briefly, 0.25 ml of lymphocyte suspension was mixed with an equal volume of either TGE viral or control antigen solution diluted 1:4. All the cultures were set up in triplicate. At 48 h of incubation at 37°C in an atmosphere of 5% CO_2 and moist air, each culture has labeled with 0.5 μCi of [^{3}H]thymidine (specific activity, 21 Ci/mmol; Radiochemical Centre, Amersham, England) for 18 h. Then incorporation of [^{3}H]thymidine

into nucleoprotein material was measured by a liquid scintillation counter. A stimulation index was defined as the proportion of the mean counts per minute of cultures incubated with TGE antigen to that of cultures incubated with control antigen.

In experiments on lymphocyte stimulation by PHA and LPS, 0.25 ml of PHA (2 μg/ml) or LPS (8 μg/ml) was added to each lymphocyte suspension.

RESULTS

Development and sequence of viral antigen-reactive cells. Background counts of cells incubated with control antigen varied with the tissue used as a cell source, but they showed similar values in the respective cell preparations in all the experiments, except those with cells collected in the acute stage of infection. Background counts of cells harvested on postinoculation day (PID) 3 to 5 (acute stage) were apparently lower than those of cells harvested in any other stage (data not shown).

No significant stimulation occurred with lymphocytes collected from any tissue of control pigs killed on PID 0 (Fig. 1). There was no difference in response between lymphocytes harvested on PID 3 to 5 and those harvested from noninfected controls. Lymphocytes obtained from all the tissues of pigs examined on PID 7 were apparently reactive to viral antigen. Thereafter, lymphocyte reactivity to the antigen increased and reached a maximal amount on PID 10 to 14.

In Peyer's patches and mesenteric lymph nodes, antigen-reactive cells were demonstrated persistently for a long time, although the stimulation index values decreased a little with the lapse of time. Lymphocytes of both tissues examined on PID 110 still responded to viral antigen. On the other hand, the proliferative reactivity of splenic and peripheral blood cells was

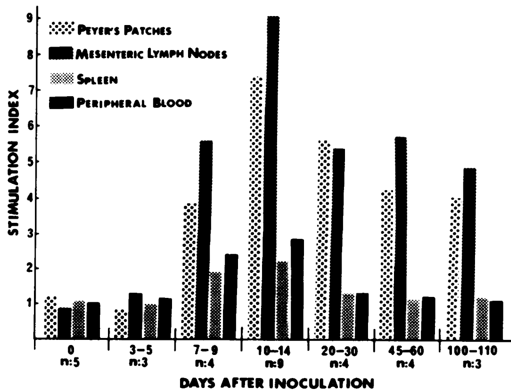

FIG. 1. *Development of lymphocytes reactive to viral antigen in pigs orally inoculated with TGE virus. n, Number of pigs examined.*

only transient. No antigen-reactive cells were detected in spleen or peripheral blood after PID 20 to 30.

Effects of ATS and complement on lymphocyte proliferative response. Cells obtained from pigs killed on PID 10 to 14 and 45 to 60 were incubated with ATS (1:80 dilution) at 37°C for 45 min and subsequently with autologous fresh serum as a complement source, then tested for proliferative reactivity. ER-forming cells were reduced greatly in number in each cell suspension after treatment with ATS and complement. Nearly the same number of cells as those capable of forming ER were killed by the treatment (Table 1). No lymphocytes treated with ATS and complement responded to PHA at all, whereas lymphocyte reactivity to LPS was slightly affected by the treatment. In pigs tested on PID 10 to 14, cells treated with ATS and complement failed to respond to TGE antigen as well as PHA. On the other hand, cells harvested on PID 45 to 60 responded a little to TGE antigen after treatment with ATS and complement, although ER-forming cells were almost completely excluded by the treatment.

Proliferative response of fractionated cells. Mesenteric lymph node cells were fractionated by the ER formation technique. The fractionated cells were tested for proliferative reactivity to TGE antigen, PHA, and LPS. The number of ER-forming cells was approximately 10 times as large in the ER-enriched fraction as in the ER-depleted fraction, whereas the number of erythrocyte-antibody-complement rosette-forming cells was larger in the latter than in the former (Table 2). Stimulation of lymphocytes by TGE antigen and PHA occurred to a greater extent in the ER-enriched fraction than in the ER-depleted fraction. On the other hand, cells were less stimulated by LPS in the ER-enriched fraction than in the ER-depleted fraction.

DISCUSSION

The proliferative response in vitro of lymphocytes to specific antigen had been considered to be one of the correlates in vitro of CMI (17). Several investigators, however, have presented evidence that viral antigen can stimulate B lymphocytes, as well as T lymphocytes, to proliferate in vitro (1, 3).

Furthermore, Moorhead (8) has reported that T lymphocytes collected from mice sensitized with 2,4-dinitrofluorobenzene can mediate delayed hypersensitivity, but do not respond to antigen in vitro, and that the reverse is true for cells of the B lymphocyte-enriched population. These previous reports seem to suggest that a

TABLE 1. *Effects of ATS and complement on the lymphocyte proliferative response*[a]

PID	Cell source[b]	Treatment	ER-forming cells (%)	Dead cells (%)	Stimulation index		
					TGE antigen	PHA	LPS
10–14	PP	ATS + complement	0	29.8	1.1	1.2	3.9
		None	13.8	12.1	6.9	27.9	4.2
	ML	ATS + complement	0.3	60.1	1.0	1.1	7.7
		None	48.5	10.1	9.5	134.3	7.8
	SP	ATS + complement	0	61.1	1.1	1.3	3.7
		None	38.5	20.5	2.1	62.5	3.8
	PB	ATS + complement	0	58.1	0.9	1.1	2.0
		None	52.6	1.8	2.5	97.3	2.1
45–60	PP	ATS + complement	0	31.2	1.7	1.1	3.3
		None	14.5	13.1	4.2	22.7	4.6
	ML	ATS + complement	0.4	58.3	1.8	1.3	5.3
		None	45.6	8.5	5.3	103.8	5.6

[a] Data are shown as averages of the results obtained from four and three pigs tested 10 to 14 days and 45 to 60 days after inoculation, respectively.

[b] PP, ML, SP, and PB indicate Peyer's patches, mesenteric lymph nodes, spleen, and peripheral blood, respectively.

TABLE 2. *Proliferative responses of populations enriched and depleted of ER-forming cells in mesenteric lymph node cells*[a]

PID	Cell fraction	ER-forming cells (%)	EACR forming cells[b] (%)	Stimulation index		
				TGE antigen	PHA	LPS
10–14	Unfractionated	49.1	30.5	10.6	153.2	8.0
	ER-enriched	85.2	7.8	17.5	198.3	2.2
	ER-depleted	8.1	73.1	3.4	20.7	9.3
45–60	Unfractionated	45.6	38.2	5.3	103.8	5.6
	ER-enriched	91.2	6.4	7.3	164.7	1.5
	ER-depleted	7.9	75.2	3.4	21.0	6.9

[a] Data are shown as average of the results obtained from three pigs each.

[b] EACR, Erythrocyte-antibody-complement rosette.

lymphocyte proliferative response to specific antigen may not necessarily be indicative of CMI in vitro.

Experiments were carried out to clarify the effects of ATS and complement on the lymphocyte proliferative response and the reactivity of fractionated cells to TGE viral antigen. Their results indicated that the lymphocyte reactivity to viral antigen in pigs orally exposed to TGE virus was largely T lymphocyte dependent. It might be possible to regard the lymphocyte proliferative response as one of the correlates in vitro of CMI in pigs infected with TGE virus. There is, however, evidence that cells harvested on PID 45 to 60 could respond a little to viral antigen even after treated with ATS and complement. This evidence indicates a possibility that B lymphocytes may also be stimulated by TGE viral antigen to proliferate in vitro in the late stage of immune responses.

Lymphocytes reactive to TGE viral antigen began to be demonstrated in all the tissues on PID 7. No differences were found in the time of appearance of antigen-reactive cells among the tissues tested. Levin et al. (7) reported that although antigen-reactive cells accumulated in Peyer's patches of rats infected with *Trichinella spiralis*, they did so not during the earliest stage of infection, as in other lymphoid tissues, but rather only in the later stage of infection. However, in the present studies, Peyer's patch cells harvested on PID 7 responded to the viral antigen like cells obtained from other tissues. This suggests that Peyer's patches participate directly in immune responses against TGE, rather than serve as a site of differentiation for immunological stem cells.

Although antigen-reactive cells were found in all the tissues tested, they varied greatly according to the type of tissue used as a cell source. They were persistently demonstrated in gut-associated lymphoid tissues (Peyer's patches and mesenteric lymph nodes), whereas the proliferative reactivity of splenic and peripheral blood cells was only transient. The persistency of antigen-reactive cells in gut-associated lymphoid tissues might be explained by the occurrence of carrier state in pigs infected with TGE virus. The existence of the carrier state in TGE has been suggested by Underdahl and co-workers (14, 15). It thus appears that lymphoid tissues associated with the intestinal tract may be given continuous or recurrent stimuli by a small amount of TGE viral antigen even after pigs have recovered from a primary TGE infection. In the early stage of infection, a portion of TGE

viral antigen may enter the circulation, since the virus replicates greatly in epithelial cells of the small intestine of infected pigs. Therefore, the viral antigen may directly stimulate not only gut-associated lymphoid tissues but also the spleen and other lymphoid tissues. Furthermore, the present studies suggests that T-lymphocyte antigen, specifically stimulated in Peyer's patches or in mesenteric lymph nodes, may not migrate into other sites, or that even if it possesses an ability to migrate into any other site, its migration may be limited to the early stage of immune response. At present, however, the efferent traffic of sensitized T lymphocytes from Peyer's patches to any other site is unknown, although it was suggested (4) that they might migrate from the intestinal tract into other sites, as do antibody-producing B lymphocytes (2).

Like the previous studies made by Frederick and Bohl (4) and Woods (18), the present studies showed clearly the occurrence of CMI in pigs inoculated with TGE virus. However, the participation of CMI in TGE infection remains obscure. Local CMI in the intestinal tract probably participates in the recovery of pigs from a primary TGE infection and in the protection of immune pigs from reinfection with TGE virus through the cytotoxic effect of sensitized T lymphocytes on newly infected target cells. Lately, it was suggested that the resistance to reinfection might be established by a cooperative action of local CMI and intestinal secretory immunoglobulin A antibody (12). We could demonstrate the presence of lymphocytes cytotoxic to TGE virus-infected target cells in pigs orally exposed to TGE virus, as reported elsewhere (M. Shimizu and Y. Shimizu, Am. J. Vet. Res., in press).

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