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STANDARDIZATION AND KINETICS OF *IN VITRO* BOVINE BLOOD LYMPHOCYTE STIMULATION WITH BOVINE ROTAVIRUS

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Abstract—Two groups of 3-month old calves were immunized intramuscularly with attenuated bovine rotavirus and boosted 21 and 42 days later. The first group of three calves were vaccinated with live virus emulsified with incomplete Freund's adjuvant (IFA) and the second group was immunized with live virus suspended in phosphate buffered saline (PBS). Three other calves, serving as controls, were inoculated with PBS emulsified with IFA. The specific cell-mediated and antibody responses of the animals were studied. Preliminary analysis of *in vitro* peripheral blood lymphocyte transformation to bovine rotavirus determined optimal conditions as: 96 h culture period, 5×10^5 cells per culture in RPMI 1640 medium containing 10% heat-inactivated bovine fetal serum and the use of inactivated virus in the cell culture at a concentration of 5×10^6 median tissue culture infective dose before inactivation. Specific blastic stimulation was observed on calves immunized with the rotavirus emulsified with IFA after the second and third vaccine inoculation with stimulation index values varying from 2.00 to 5.73. Serum neutralizing antibody titers of 1/25,600 were also induced in the same calves. Calves immunized with rotavirus-PBS suspension developed a mean antibody titer of 1/1,600, but showed no specific lymphocyte stimulation. No increase in specific immune responses was detected in the control animals.

Key words: Bovine rotavirus, lymphocyte stimulation test, cellular immunity, neutralizing antibodies, vaccination, adjuvant

Résumé—Deux groupes de veaux âgés de 3 mois furent vaccinés par la voie intramusculaire en utilisant une souche vivante atténuée de rotavirus bovin, avec des doses de rappel aux jours 21 et 42 après la première immunisation. Un premier groupe de trois veaux fut immunisé avec le virus incorporé dans une émulsion eau dans l'huile, préparée avec l'adjuvant incomplet de Freund (AIF), alors que les animaux du deuxième groupe furent vaccinés avec le virus mélangé avec un tampon phosphate salin (PBS). Trois veaux témoins furent inoculés avec le PBS incorporé dans l'AIF. Les cinétiques des réponses spécifiques de l'immunité à médiation cellulaire et des anticorps des animaux furent étudiées. Une étude préliminaire sur la stimulation *in vitro* des lymphocytes du sang par le rotavirus bovin avait permis d'établir les conditions optimales pour la réalisation du test: une incubation de 96 h des cultures, une quantité de 5×10^5 cellules par culture en utilisant le milieu RPMI 1640, additionné de 10% de sérum foetal bovin et l'utilisation de virus inactivé à une concentration de 5×10^6 particules virales, produisant un effet cytopathique sur 50% des cultures cellulaires infectées avant l'inactivation. Une stimulation lymphoblastique spécifique fut observée chez les veaux immunisés avec le virus et l'AIF après les deuxième et troisième doses vaccinales, les indices de stimulation variant de 2,00 à 5,73. Ces derniers veaux ont aussi montré des titres sériques en anticorps neutralisants de 1/25.600. Les veaux vaccinés avec le virus et le PBS ont aussi développé une réponse en anticorps avec un titre moyen de 1/1.600, sans manifester toutefois une stimulation lymphoblastique spécifique. Les veaux témoins n'ont démontré aucune augmentation des réponses immunitaires spécifiques.

Mots-clefs: Rotavirus bovin, test de stimulation lymphoblastique, immunité cellulaire, anticorps neutralisants, vaccination, adjuvant

INTRODUCTION

Rotavirus is considered as a main agent incriminated in diarrhea in children and newborn animals [1–3]. In calves, diarrhea due to rotavirus appears mostly in the first 2 wk of life [2, 3]. The induction of active immunity, both locally in the intestine and systemically, following oral vaccination or experimental infection of the calf, has been reported [4–8]. For instance, orally vaccinated calves challenged 48–72 h after vaccination remained normal and showed a significant neutralizing antibody response in the intestine and in the serum [5, 6]. Bachmann *et al.* [4] detected specific antibodies in the jejunum and in the feces 2–12 days and 4–8 days respectively following the rotavirus infection. A similar observation was also reported by Van Zaane *et al.* [7] who detected specific fecal antibodies of IgM and IgA classes 5 and 10 days respectively, after the rotavirus infection. Another study [8] showed that inoculation of attenuated bovine rotavirus either orally or directly into an isolated intestinal loop induced the appearance of rotavirus antibody-producing cells located in the mucosa of the proximal small intestine with a local production of specific immunoglobulins mainly of the IgA class.

The passive protection of the calves against rotavirus infection may be obtained by immunizing the dams during the last trimester of pregnancy with adjuvanted rotavirus [9–12]. The specific antirotaviral antibodies, mainly IgG_1, present in the colostrum and milk of the vaccinated cows were shown responsible for that passive protection. On the other hand, cell mediated immunity (CMI) is considered important in the protection and recovery of certain viral infections [13–15]. The lymphocyte stimulation test (LST) which is a blastic transformation in response to the mitogens or antigens including the infectious agents is used as an *in vitro* correlate of CMI in animals and humans [16, 17]. The purpose of the present study is to report the standardization and application of the LST as an *in vitro* correlate of CMI in the bovine following systemic immunization with adjuvanted bovine rotavirus.

MATERIALS AND METHODS

Cell culture

The African Green Monkey kidney cells (BSC-1) were propagated as monolayers at 37°C in 150 cm^2 plastic culture flasks (Corning Glass Works, New York), using Eagle's minimum essential medium with Earle's salt and L-glutamine (EMEM) [Flow Laboratories, Rockville, Maryland], supplemented with 10% heat-inactivated fetal bovine serum (FBS) [Gibco Laboratories, Chagrin Falls, Ohio], 100 IU/ml of penicillin and 100 μg/ml of streptomycin (Flow Laboratories). For virus production, infected cells were maintained in EMEM supplemented with 5 μg/ml of bovine crystallized trypsin [Sigma Chemical Company, St Louis, Missouri] (EMEM-T) and antibiotics.

Virus production

The rotavirus used in this study was the plaque-purified Neonatal Calf Diarrhea Virus (NCDV) strain [18, 19]. Viruses were produced as previously described [18] and were used for animal vaccination and in the LST. When 70–90% of the cells exhibited a cytopathic effect (CPE), virus was harvested by osmotic shock followed by a trichlorofluoroethane [Freon 113] (Dupont of Canada Limited, Maitland, Ontario) extraction [20]. The viruses were then purified by CsCl isopycnic gradient ultra-centrifugation as described by Fauvel

et al. [20]. After centrifugation at 110,000 *g* for 3 h, fractions ranging from 1.34 to 1.39 g/ml in CsCl and in which the viral bands at 1.36 and 1.38 g/ml in CsCl are located [21], were collected and dialyzed overnight against 0.05 M Tris–HCl buffer, pH 8.0. The viral titers were determined and calculated in median tissue culture infective dose ($TCID_{50}$) per ml as described [22, 23].

Animal vaccination

Three-month old male Holstein–Freisian calves, with no or negligible rotaviral serum antibody titers by neutralization test ($< 1/25$), were kept in an isolated barn with controlled temperature and ventilation. The animals were allowed to adapt to their surroundings for 3 wk before starting the experiment. For the optimization of the specific LST, two calves (T010 and 85) were inoculated intramuscularly every 3 wk in the two semi-tendinous and semi-membranous regions with 5 ml of a mixture (2.5 ml per site) containing 2.5 ml of live purified rotavirus suspension (2×10^8 $TCID_{50}$/ml) emulsified with 2.5 ml of incomplete Freund's adjuvant (IFA) [Gibco Laboratories]. For the kinetic studies of the immune responses, three animals were vaccinated with the emulsion containing the virus and IFA and boosted twice 21 and 42 days after the first vaccine inoculation. Another group of three animals were inoculated with 5 ml of a mixture containing the rotavirus suspension mixed in equal volume with phosphate buffered saline (PBS). Three other animals were inoculated with PBS emulsified in IFA and served as the control group.

Preparation of the antigen for the in vitro lymphocyte stimulation test

The virus used as specific antigen in the LST was inactivated by the method previously described by Bahnemann [24]. Briefly, 0.3 ml of the purified viral suspension which had a titer of 2×10^9 $TCID_{50}$/ml before inactivation and 55 μg/ml of protein content as determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, California) was mixed with 2.1 ml of PBS and 0.3 ml of 0.1 M 2-bromoethylamine (BEA) hydrobromide (Sigma Chemical Company), pH 7.5, previously activated with NaOH 0.2 N for 1 h at 37°C. This mixture was incubated for 4 h at 37°C. Thereafter, 0.3 ml of 20% cold sodium thiosulfate was added to stop the inactivation reaction. The inactivated viral suspension was dialyzed for 36 h against three changes of the same culture medium used in the LST without the FBS. The viral antigen was checked for any residual infectivity on BSC-1 cells and was kept in small aliquots at −70°C. A non-infected cell lysate antigen was similarly prepared from a fraction of 1.34 to 1.39 g/ml and used as the control. Both preparations were checked for the presence of endotoxin content by the limulus lysate method (LLM) [25], using a commercial kit (Pyrotest, Difco Laboratories, Detroit, Michigan).

Preparation of lymphocyte suspension

10 ml of peripheral blood were collected weekly for up to 70 days after the first vaccination dose by jugular venipuncture and placed into tubes containing 1 ml of 0.15 M sodium citrate as an anticoagulant. The blood was mixed with an equal volume of PBS containing 10% (v/v) of 0.15 M sodium citrate (PBS-citrate) and buffy coat cells were obtained by centrifugation at room temperature for 20 min at 400 *g*. Mononuclear cells were obtained by placing 8 ml of a mixture of buffy coat cells and diluted autologous plasma on 5 ml of Ficoll-Paque (Pharmacia, Uppsala, Sweden), density 1.076 g/ml, and centrifuging at 600 *g* for 30 min. Cells recovered from the interface were washed 3 times

with PBS-citrate and resuspended to the desired concentration. The cells collected had a viability of more than 95% by the trypan blue dye exclusion method. Smears stained with Wright solution showed that 88–94% of the cells were lymphocytes.

In vitro lymphocyte stimulation test

For optimization of the bovine rotavirus LST, the isolated cells were resuspended at different concentrations (1.25, 2.0 and 2.5 × 10^6 cells/ml) in RPMI 1640 medium (Flow Laboratories), supplemented with 10% of FBS, 2 mmol of L-glutamine (Flow Laboratories) and antibiotics. The cell culture medium was shown free of endotoxin before used, as tested by the LLM. The cell suspension (200 μl) was added to each well of 96 flat-bottomed well microtiter plates (Flow Laboratories). Quadruplicate wells received 25 μl of either the viral antigen used at different concentrations or the cell lysate antigen control and the lymphocyte cultures were incubated for 2, 3 or 4 days at 37°C in a 5% CO_2 humidified atmosphere. Stimulation of the cells by the Concanavalin A (Con A) [Difco Laboratories] used at optimal concentration was always done for each cell sample using a concentration of 1.25 × 10^6 cells/ml and 3 days of incubation for the test [17].

All lymphocyte cultures were pulsed with 1 μCi of tritiated thymidine (sp. act. of 6.7 Ci/mmol) [New England Nuclear, Boston, Massachusetts] diluted in 25 μl of the culture medium without FBS, 20 h before harvesting onto filter paper disks with a semi-automated multiple cell harvester (Flow Laboratories). The dried filter paper disks were then transferred into 5 ml vials and solubilized with 3.5 ml of Aquasol (New England Nuclear) scintillation fluid for 45 min and the vials were counted for 2 min in a scintillation spectrometer (Model LS 58, Beckman Instruments Inc., Palo Alto, California). The data were expressed in counts per minute (cpm) and in calculating the stimulation index (SI), which represents the ratio of cpm incorporated by the virus containing cultures to the cpm incorporated by the control cultures. The LST was significant at SI $\geqslant$ 2.00 [16].

Virus neutralization test

The presence of specific neutralizing antibodies was carried out on sera obtained from the calves before vaccination (day 0) and at day 14, 28, 49 and 70 after the first vaccine inoculation. For testing, the sera were inactivated at 56°C for 30 min. Serial 2-fold dilutions (starting at 1/25) of each serum in EMEM-T were mixed with an equal volume of 0.1 ml (8,000 $TCID_{50}$) of the rotavirus and incubated at 37°C for 1 h. The residual infectivity was tested in quadruplicate using confluent BSC-1 cells in 96 well microtiter plates. A volume of 25 μl of each serum–virus mixture was placed in each well and an incubation of 75 min at 37°C was allowed. The inoculum was removed and 200 μl of EMEM-T were added to each well and the plates were incubated at 37°C and 5% CO_2. Final readings were made on the fourth day when the CPE was complete in the virus control cultures. The antibody titer of each serum was expressed as the highest reciprocal dilution neutralizing 1,000 $TCID_{50}$ of the virus.

RESULTS

Standardization of the lymphocyte stimulation test specific to bovine rotavirus

Parameters such as the number of cells per culture, incubation time and viral concentration were analysed from data collected 2 wk after the third vaccine inoculation of calves T010 and 85. Table 1 shows that a concentration of 2.5 × 10^6 mononuclear

Table 1. Effect of cell concentration on *in vitro* lymphocyte stimulation by bovine rotavirus[a]

Calf	Number of cells per culture	Control cells		Stimulated cells		
		Mean cpm[b]	SD	Mean cpm	SD	SI[c]
T010	250,000	2,842	455	4,578	780	1.61
	400,000	3,986	472	10,006	1,109	2.51
	500,000	4,482	719	16,178	2,106	3.61
85	250,000	3,242	388	6,354	483	1.96
	400,000	4,692	420	13,982	977	2.98
	500,000	6.284	556	23,376	1,687	3.72

[a] 5×10^6 ($TCID_{50}$) inactivated viral particles per culture and 4 days of incubation.
[b] Mean counts per min of 4 cultures of lymphocytes.
[c] Stimulation index.

Table 2. Effect of incubation time on *in vitro* lymphocyte stimulation by bovine rotavirus[a]

Calf	Incubation time (days)	Control cells		Stimulated cells		
		Mean cpm[b]	SD	Mean cpm	SD	SI[c]
T010	3	8,895	947	29,276	2,827	3.29
	4	4,482	719	16,178	2,016	3.61
	5	4,200	132	8,634	603	2.01
85	3	9,897	682	27,909	1,112	2.82
	4	6,284	556	23,376	1,687	3.72
	5	5,486	682	10,094	882	1.84

[a] 5×10^5 cells and 5×10^6 ($TCID_{50}$) inactivated viral particles per culture.
[b] Mean counts per min of 4 cultures of lymphocytes.,
[c] Stimulation index.

Table 3. Effect of viral concentration on *in vitro* lymphocyte stimulation by bovine rotavirus[a]

Calf	Control cells		Stimulated cells			
	Mean cpm[b]	SD	Number of inactivated viral particles per culture ($TCID_{50}$)	Mean cpm	SD	SI[c]
T010	4,398	191	5×10^6	17,177	670	3.90
			5×10^5	11,926	964	2.71
			5×10^4	7,981	320	1.81
85	2,466	461	5×10^6	8,499	617	3.44
			5×10^5	6,222	438	2.52
			5×10^4	3,372	539	1.37

[a] 5×10^5 cells per culture and 4 days of incubation.
[b] Mean counts per min of 4 cultures of lymphocytes.
[c] Stimulation index.

cells/ml (5×10^5 cells per culture) provided maximal lymphocyte stimulation. A concentration of 2×10^6 cells/ml (4×10^5 cells per culture) was still sufficient to induce a specific stimulation while no significant stimulation (SI < 2.00) resulted from the cultures containing 2.5×10^5 mononuclear cells. As shown in Table 2, the length of incubation time had also an effect on the LST. Even if the mean cpm for control and stimulated cells were higher for the cultures incubated for 3 days, the best results as indicated by the SI values were obtained after an incubation of 4 days. Significant stimulation was also induced after 3 days of incubation for both calves and 5 days of incubation for calf T010.

As reported in Table 3, a minimum of 5×10^5 $TCID_{50}$ inactivated viral particles per lymphocyte culture was necessary to induce a significant lymphocyte stimulation. Maximal results were obtained with 5×10^6 $TCID_{50}$ inactivated viral particles indicating an approximate ratio per culture of ten viral particles for each mononuclear cell. No

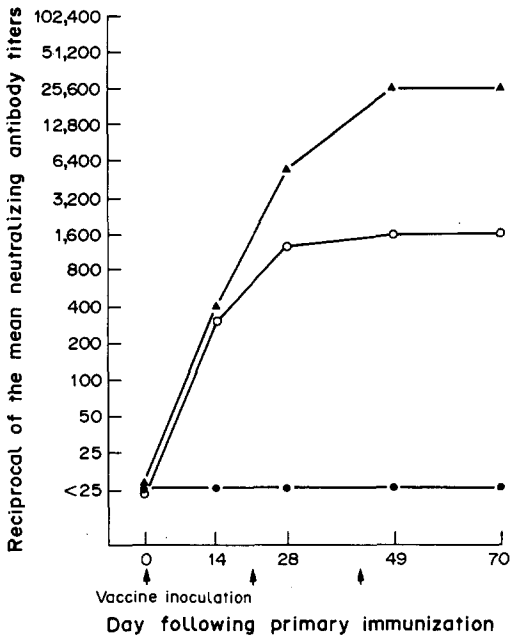

Fig. 1. Kinetics of specific neutralizing antibodies in serum of calves vaccinated with bovine rotavirus. ●——● control group (PBS and IFA); ○——○ rotavirus and PBS; ▲——▲ rotavirus and IFA.

stimulation was shown using the lower concentration of 5×10^4 $TCID_{50}$ viral particles per culture.

Based on these results, conditions used subsequently in the bovine rotavirus LST included the use of 5×10^5 lymphocytes per well, 5×10^6 $TCID_{50}$ inactivated viral particles per well, and an incubation period of 4 days.

Immune response of vaccinated calves

The kinetics of specific neutralizing antibodies in serum of each group of calves are presented in Fig. 1. A significant immune response was obtained as early as 14 days after the first vaccine inoculation for the two groups immunized with the virus. Anamnestic response was observed for both groups after the booster inoculation given at day 21 post-primary immunization (PPI). The group vaccinated with the rotaviral water-in-oil emulsion showed a mean neutralizing antibody titer of 1/25,600 by day 49 following PPI. The other group vaccinated with the aqueous rotaviral suspension has demonstrated an intermediate response reaching a mean antibody titer of 1/1,600. A significant difference ($P < 0.001$) by a Student's t-test between antibody titers of the two rotaviral vaccinated groups was observed at day 49 PPI. For the control group inoculated with the emulsion without the rotavirus, the antibody titers remained at the minimal level ($<1/25$) throughout the experiment.

Figure 2 represents the kinetics of peripheral blood lymphocytes stimulated *in vitro* with the bovine rotavirus. Neither the calves of the control group (Fig. 2A) nor those vaccinated with the aqueous rotaviral suspension (Fig. 2B) showed any specific lymphocyte stimulation during the experiment. Vaccination of the calves with the rotavirus emulsified in IFA

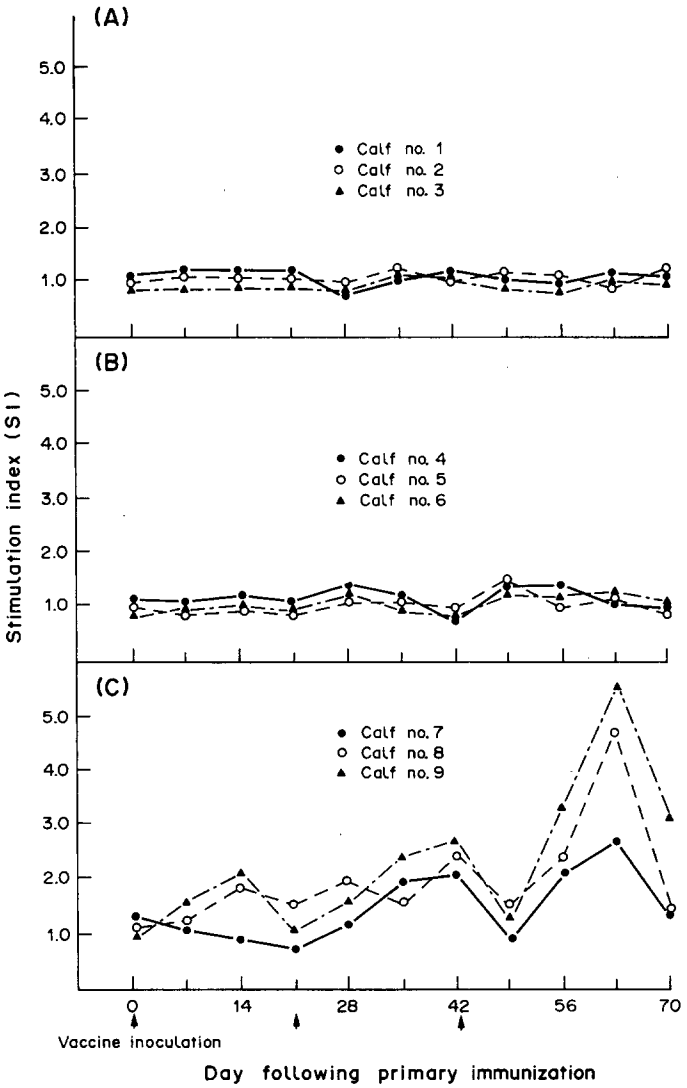

Fig. 2. Kinetics of specific *in vitro* lymphocyte activity following immunization of calves with bovine rotavirus. A: control group (PBS and IFA); B: rotavirus and PBS; C: rotavirus and IFA.

resulted in a specific lymphocyte activity with significant SI varying from 2.00 to 5.73 (Fig. 2C). It also showed two peaks of stimulation after the second and third vaccine inoculation.

DISCUSSION

The LST is an important assay for evaluating the immunologic competence of animals and the specific immune response to different antigens [14]. In the present paper, the LST was used to investigate the CMI response following intramuscular vaccination of calves with rotavirus.

For standardization of the LST with bovine rotavirus, different parameters were analysed. Optimal results were obtained after 4 days of incubation with 5×10^5 lymphocytes per culture stimulated with 5×10^6 $TCID_{50}$ inactivated viral particles. A lower lymphocyte specific activity, as determined by the SI values after 5 or 3 days of cell culture incubation, was probably due to a diminution in cell nutrients resulting in mortality of the cells or a shorter time of antigenic recognition by the lymphocytes respectively. However, the possible presence of suppressive cells or molecules such as prostaglandins [17] in the cell cultures might also explain that lower lymphocyte activity. A good correlation was also obtained for the SI values and the increasing number of the cells in each culture, enhancing the probability of contact between the sensitized lymphocytes and the antigen. The ratio of 10 viral particles for each mononuclear cell gave the best results in this study. However, in one experiment, higher ratio of 20 viral particles for each cell (5×10^6 viral particles vs 2.5×10^5 cells per culture) [Table 1] did not contribute to a significant augmentation of the SI. The use of inactivated virus particles as specific antigen in the LST was chosen instead of the live virus since it has been reported in humans that rotaviruses may infect leukocytes under mitogen stimulation [26], with a potential abrogation of lymphocyte metabolism or of cell mortality.

An important fluctuation of the control cell culture cpm (background) was observed among individual calves and also on the same calves during time-course studies (data not shown). Indeed, most of the mean cpm for the control cell cultures varied from 1,500 to 10,000, as for calves T010 and 85, which is similar to the results reported by other authors in the bovine [27, 28]. The stimulation of the cells by the Con A, which is primarily mitogenic for T lymphocytes in many species including the bovine [28], was performed to check the viability and the functional activity of the isolated cells.

Vaccination of the calves with the IFA adjuvanted bovine rotavirus induced a diphasic specific lymphocyte transformation, which has been previously described in the bovine, even after a single vaccine inoculation [29]. Furthermore, the specific *in vitro* lymphocyte stimulation coincided with maximal antibody titers in the serum of the same calves. The need for more than one immunization correlated with the secondary immune response which was obtained after the booster vaccine inoculations. As it was demonstrated in other studies in the bovine species [10, 11], the use of IFA as adjuvant was satisfactory to induce a significant humoral immune response following systemic immunization with bovine rotavirus. The increased antibody formation that generally follows the inoculation of the antigen incorporated into a water-in-oil emulsion is recognized to be associated with the antigen-depot effect as a means of greater persistence of the antigen *in vivo*, thus extending the period of antigenic stimulation, and to the attraction of immunocompetent cells to the area of injection [30].

The importance of CMI and its defense role were demonstrated in a few viral infections [13–15]. For rotavirus infection, it has been demonstrated that optimal CMI as measured by LST with splenic lymphocytes correlates with the end of virus replication in the intestine and follows the termination of diarrhea in young infected mice [31]. Another report [32] showed that delayed-type hypersensitivity (DTH) elicited by subcutaneous inoculation of murine rotavirus was observed in neonatal (12-day old) and adult (>30-day old) mice respectively 21 and 7 to 10 days following rotavirus infection orally. This DTH response was however not seen in neonatal mice when infected at 5 days of age. Another study [33] has reported the ability of simian rotavirus-stimulated human peripheral blood leukocytes to produce interferon and cytokines, which stimulated the killing *in vitro* of rotavirus-

infected cells by a second set of leukocytes described as natural killer cells. As reported in this study, immunization of calves with adjuvanted bovine rotavirus induced an *in vitro* specific proliferative activity of peripheral blood lymphocytes. However, the role of these lymphocytes sensitized to bovine rotavirus remains to be determined.

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