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Rotavirus induces proliferative response and augments non-specific cytotoxic activity of lymphocytes in humans

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SUMMARY

In vitro cell-mediated immune responses to rotavirus in humans were studied. Peripheral blood mononuclear cells (PBMC) of healthy adults proliferated in response to stimulation with the infectious and u.v.-inactivated Wa strain of human rotavirus, showing a maximum response on day 7 of culture; however, cord blood lymphocytes failed to respond to rotavirus. A cross-reactive proliferative response of PBMC detected by stimulation with the NCDV strain of bovine rotavirus suggests the existence of epitopes common to both human and bovine rotaviruses, which are recognized by human T lymphocytes. The phenotype of the majority of activated lymphocytes was $CD3^+4^+8^-$, indicating that the cells mainly activated were helper T cells. Culture supernatants of PBMC stimulated with rotavirus contained interleukin-2 (IL-2) and interferon-gamma (IFN-γ). In addition, PBMC stimulated with rotavirus demonstrated significantly enhanced cytotoxic activity against natural killer (NK) sensitive K562 cells as well as an NK-resistant Epstein–Barr virus-immortalized lymphoblastoid cell line (LCL). Treatment of PBMC with anti-CD16 or NKH1A monoclonal antibody, both of which react with most NK cells and lymphokine-activated killer cells and complement markedly reduced the cytotoxic activity against K562 and LCL. These results suggest that stimulation of human PBMC with rotavirus results in the production of lymphokines, such as IL-2 and IFN-γ, by rotavirus-reactive helper T cells and that these lymphokines augment NK activity and generate other forms of non-specific cytotoxic human lymphocyte activity. These cell-mediated immune responses observed in the present *in vitro* study might play an important role in protection and recovery from rotavirus infection.

Keywords rotavirus cellular immunity cytotoxicity proliferative response

INTRODUCTION

Rotavirus is a major cause of serious acute gastroenteritis in infants and young children, causing approximately one-million diarrhoeal deaths every year in developing countries (Kapikian & Chanock, 1985; Flores *et al.*, 1986). In order to prevent severe rotavirus diarrhoea, there is an urgent need to develop effective rotavirus vaccines. Candidates for rotavirus vaccines that have been evaluated in clinical trials include bovine NCDV (RIT4237), bovine WC3 and Rhesus RRV (MMU18006) strains (Vesikari *et al.*, 1985; Flores *et al.*, 1987; Clark *et al.*, 1988). Some candidates marked high efficacy when they were administered to older infants (Vesikari *et al.*, 1984, 1985), but none of them appeared to confer protection against all human serotypes when they were given to younger infants. Experience in vaccine trials, therefore, suggests a need to understand fully the mechanisms of human immune response to rotavirus infection. Although recent studies have indicated potentially important protective roles of substances present in breast milk (Schoub *et al.*, 1977), antibodies in serum and locally produced intestinal tract antibodies (Sack *et al.*, 1980; Riepenhoff-Talty *et al.*, 1981), the prime protective factors involved in immunity to rotavirus infection have yet to be determined conclusively.

The production of antibodies by B cells depends on and is controlled by various forms of cell-mediated immunity. Therefore, it seems important to study the cellular immune response in order to clarify the mechanisms of protection against rotavirus infection. In addition, the finding that high titres of circulating antibodies do not protect against rotavirus infection in a murine experimental system (Offit & Clark, 1985) suggests that cell-mediated immune responses may also play a direct role in protection and recovery from rotavirus infection. Although the humoral immune responses to rotavirus in humans have been well studied, the precise mechanisms of cell-mediated immune responses to rotavirus infection in humans have not been clarified. The present study was therefore undertaken to determine whether peripheral blood lymphocytes of adults show

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a secondary response to rotavirus *in vitro* and to clarify the characteristics of rotavirus-reactive human lymphocytes. We report here that peripheral blood mononuclear cells (PBMC) of the vast majority of adults showed a proliferative response to rotavirus along with production of lymphokines, such as interleukin-2 (IL-2) and interferon-gamma (IFN-γ). Furthermore, it was found that stimulation of human lymphocytes with rotavirus resulted in augmentation of non-specific cytotoxic activity, possibly induced by the lymphokines produced by rotavirus-reactive helper T cells. The circuit of cell-mediated immune response to rotavirus observed in the present study might have an important role in limiting the spread of rotavirus infection *in vivo*.

MATERIALS AND METHODS

Viruses and preparation of virus antigens

The Wa strain (serotype 1) of human rotavirus and the NCDV strain (serotype 6) of bovine rotavirus were grown in MA104 cells, a cell line derived from Rhesus monkey kidney, as described previously (Nakogomi *et al.*, 1987). Infectious titre of virus was determined by a plaque assay as described previously (Hoshino *et al.*, 1984). The inactivation of viral preparations was performed by exposure to u.v. irradiation from a 15-W u.v. light-bulb for 10 min at a distance of 10 cm. After u.v. irradiation, no viral plaques were detected on an MA104 monolayer.

Cell lines

MJ-LCL was established in our laboratory by infecting human B cells with Epstein–Barr virus as described previously (Sugden & Mark, 1977). MJ-LCL and the NK-sensitive cell line K562, which was established from a patient with chronic myelogenous leukaemia (Lozzio & Lozzio, 1975), were grown in RPMI 1640 medium supplemented with 10 mM HEPES buffer and 10% heat-inactivated fetal calf serum (FCS) (GIBCO, Grand Island, NY).

Monoclonal antibodies

OKT8 (anti-CD8), Leu11b (anti-CD16) and NKH1A used for complement-dependent cell selection were purchased from Ortho Pharmaceutical Corp. (Raritan, NJ), Becton Dickinson (Mountain View, CA) and Coulter (Hialeah, FL), respectively. FITC-conjugated Leu4 (anti-CD3), Leu3a (anti-CD4), Leu2a (anti-CD8), IL-2R (anti-CD25) and anti-HLA-DR monoclonal antibodies used for immunofluorescence flow cytometry were purchased from Becton Dickinson.

Cell separation and treatment of cells with monoclonal antibodies and complement

PBMC from rotavirus-seropositive healthy adults and cord blood lymphocytes from a healthy full-term baby were isolated by Ficoll–Conray gradient centrifugation. Freshly separated PBMC or rotavirus-stimulated PBMC were suspended at a concentration of 20×10^6 cells/ml in RPMI 1640 medium supplemented with 10 mM HEPES buffer and 10% heat-inactivated pooled human **AB** serum (referred to hereafter as culture medium) containing 50 μl of monoclonal antibody. The cells to be treated with complement alone were incubated in culture medium without antibody. After 30 min of incubation on ice, non-toxic rabbit complement (Cedarlane, Hornby, Canada) was added at a final dilution of 1:4, and the cells were incubated for 1 h at 37°C. The cells were then washed twice and resuspended in culture medium.

Stimulation of cells with rotavirus

Ten-million PBMC or monoclonal antibody-treated cells were suspended in 20 ml of culture medium, then 1×10^6 plaque-forming units (PFU) of human rotavirus were added to the cells. The cells were cultured in an upright flask (Falcon 3013) at 37°C in a 5% CO_2 incubator for 7 days.

Proliferative response to rotavirus

PBMC and cord blood lymphocytes were cultured at 1×10^5/200 μl in culture medium in round-bottomed 96-well microtitre plates (Nunc 163320) with various dilutions of infectious or u.v.-light-irradiated rotavirus. The plates were incubated at 37°C in a 5% CO_2 incubator for 7 days. For the final 12 h of incubation, 1 μCi of ^{3}H-thymidine (^{3}H-TdR) (New England Nuclear, Boston, MA) was added, and the cells were then harvested onto glass filter paper by a semi-automatic multiple cell harvester. The incorporation of ^{3}H-TdR was determined by liquid scintillation counting. In the kinetics study, cultures were performed for various periods and the incorporation of ^{3}H-TdR was determined as described above.

Immunofluorescence flow cytometry

Five-hundred thousand cells were incubated with 100 μl of phosphate-buffered saline (PBS) containing 5% human immunoglobulin (Hoechst Japan, Tokyo, Japan) for 15 min at 4°C to block the Fc receptors on the cell surface. The cells were then incubated with 10 μl of fluorescein-conjugated monoclonal antibodies for 30 min at 4°C. After washing three times with PBS containing 2% FCS, the cells were analysed with an Epics profile flow cytometer (Coulter).

Cytotoxic assays

^{51}Cr-release assays were performed as described previously (Yasukawa & Zarling, 1984a). Briefly, target cells, K562 or MJ-LCL, were incubated for 1 h at 37°C with 100 μCi ^{51}Cr ($Na_2{}^{51}CrO_4$) (New England Nuclear) and then washed twice with cold culture medium. Various numbers of effector cells and 1×10^4 ^{51}Cr-labelled target cells were incubated together in 200 μl of culture medium in round-bottomed microtitre wells. Target cells were also added to wells containing medium alone and to wells containing detergent to determine the spontaneous and maximal release, respectively. After 5 h, 100 μl of supernatant were removed from each well and transferred to tubes for counting in a gamma counter. The percentage of specific ^{51}Cr release (ct/min) was calculated as follows:

$$\frac{\text{Experimental} - \text{Spontaneous release}}{\text{Maximal} - \text{Spontaneous release}} \times 100$$

The spontaneous release from the target cells never exceeded 15% of the maximal release.

Assays for production of IL-2 and IFN-γ

Two-million PBMC suspended in 2 ml of culture medium were cultured in a 16-mm well with human rotavirus at a final concentration of 1×10^5 PFU/ml or without rotavirus. The cultures were performed for various periods, and culture supernatants were collected and stored at −20°C until used for

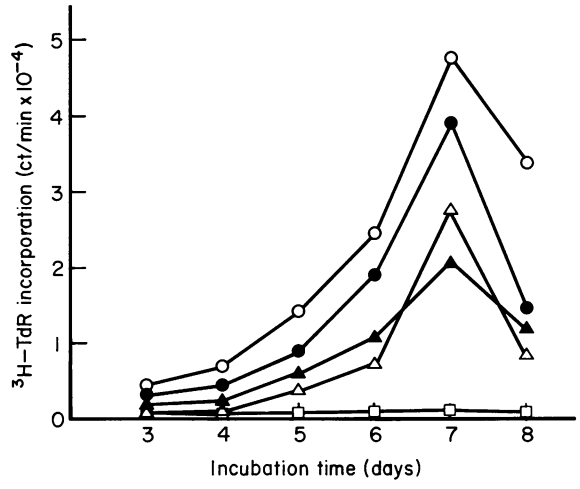

Fig. 1. Kinetics of lymphocyte proliferative response mediated by stimulation with rotavirus. PBMC isolated from four healthy adults (○, ●, △, ▲) and cord blood lymphocytes isolated from a healthy fullterm baby (□) were cultured with native human rotavirus at a concentration of 1×10^5 PFU/ml for 3, 4, 5, 6, 7 and 8 days. Incorporation of ^{3}H-TdR was determined during the final 16 h of culture. The results are mean ct/min for triplicate wells.

assays. The assay for IL-2 activity in culture supernatants was performed as described previously (Yasukawa & Zarling, 1984b). The CTLL cell line, an IL-2-dependent line (Gillis & Smith, 1977), was used as the indicator cells for IL-2 activity. CTLL cells (1×10^4) in 100 μl of RPMI 1640 medium supplemented with 10% FCS were plated in flat-bottomed microtitre wells (Falcon 3072), and then 100 μl of culture supernatant were added to each well. The CTLL cells were cultured for 30 h at 37°C in a 5% CO_2 incubator. For the final 6 h of incubation, 1 μCi of ^{3}H-TdR was added, and the incorporation of ^{3}H-TdR was measured as described above. Assay for IFN-γ production in culture supernatants was performed by radioimmunoassay using a monoclonal antibody to IFN-γ (Centocor, Malvern, PA).

RESULTS

Kinetics of lymphocyte proliferative response to stimulation with rotavirus

We first addressed the question of whether lymphocytes from adults respond to stimulation with rotavirus *in vitro*. Representative data from kinetics studies using PBMC from four healthy adults and cord blood lymphocytes from a healthy full-term baby are shown in Fig. 1. PBMC showed evident proliferation in response to stimulation with human rotavirus, and the maximum response was detected at day 7 of culture. Incorporation of ^{3}H-TdR into lymphocytes stimulated with mock-infected MA104 prepared by the same procedure as that for rotavirus antigen never exceeded 1000 ct/min (data not shown). Similar results were obtained using PBMC from four other donors, and PBMC from a single donor separated at different times (data not shown). In contrast, no proliferative response was detected when cord blood lymphocytes were cultured with rotavirus. These data suggest that the response is not mediated via an antigen-non-specific mitogenic effect of rotavirus, but rather occurs in an antigen-specific manner.

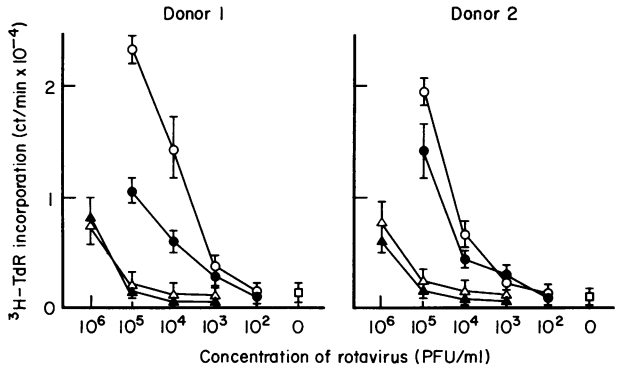

Fig. 2. Proliferative response of human lymphocytes mediated by stimulation with human or bovine rotavirus at various concentrations. PBMC isolated from two healthy adults were cultured with the native Wa strain of human rotavirus (○), the u.v.-light-treated Wa strain of human rotavirus (●), the native NCDV strain of bovine rotavirus (△) or the u.v.-light-treated NCDV strain of bovine rotavirus (▲) for 7 days. Incorporation of ^{3}H-TdR was determined during the final 16 h of culture. The results are mean (ct/min) s.e.m. for triplicate wells.

Proliferative response of lymphocytes stimulated with various concentrations of human and bovine rotaviruses treated and untreated by u.v. light

Dose–response curves of the proliferative responses mediated by human and bovine rotavirus antigens were determined by culturing lymphocytes from two healthy individuals with various concentrations of the rotavirus antigens. Because of the relatively low titre of the human rotavirus preparation, we were unable to examine the response of lymphocytes to human rotavirus at more than 1×10^5 PFU/ml. Within the range of antigen concentration examined, maximum response to human and bovine rotavirus were detected at 1×10^5 and 1×10^6 PFU/ml, respectively. The response of human lymphocytes to bovine rotavirus suggests that human and bovine rotaviruses share some common antigenic epitopes recognized by T lymphocytes. We also examined the effect of virus infectiousness on the proliferative response using u.v.-treated and untreated rotaviruses. As shown in Fig. 2, although the response to native rotavirus was somewhat higher than that to u.v.-treated rotavirus, the inactivated rotavirus was also capable of stimulating lymphocytes.

Phenotype of lymphocytes activated by stimulation with rotavirus

We next examined the phenotype of activated lymphocytes cultured with rotavirus using a flow cytometer by adjusting the bitmap gate for only large lymphoblasts. The cell surface phenotypes of activated lymphocytes from two individuals are shown in Table 1. Although CD4$^+$ and CD8$^+$ lymphocytes were both activated by stimulation with rotavirus, CD4$^+$ T lymphocytes were predominant among the blasts generated. These data suggest that although various lymphocyte subpopulations are capable of responding to rotavirus, CD4$^+$ T lymphocytes are mainly activated, indicating that helper T cells are the major cell population involved.

Augmented nonspecific cytotoxicity of lymphocytes stimulated with rotavirus

Since NK activity and lymphokine-activated killer (LAK) activity are considered to constitute one of the most important

Table 1. Phenotype of blasts generated by stimulation with rotavirus

Donor	Cells	Phenotype CD3	CD4	CD8	CD25	Ia
1	Fresh PBMC	72	48	21	2	10
	Rotavirus-stimulated PBMC	84	70	12	70	85
2	Fresh PBMC	70	42	23	5	12
	Rotavirus-stimulated PBMC	82	68	13	81	86

Phenotypes of blasts generated by stimulation with rotavirus and freshly separated PBMC were determined by direct immunofluorescence assay using a flow cytometer. Results are expressed as percentages of positive cells.

Table 2. Effect of stimulation of lymphocytes with rotavirus on cytotoxicity against K562 and LCL

Cells	% specific ^{51}Cr release from target cells* K562 40:1	20:1	10:1	MJ-LCL 40:1	20:1	10:1
Donor 1						
Fresh PBMC	36·2	30·5	15·6	2·3	1·5	0·1
PBMC+mock	20·5	15·2	12·0	3·6	2·2	1·9
PBMC+rotavirus	62·3	54·2	42·8	34·3	27·6	18·8
Donor 2						
Fresh PBMC	40·9	35·5	19·2	3·5	2·9	2·0
PBMC+mock	32·3	19·8	10·2	5·2	4·3	2·5
PBMC+rotavirus	70·5	67·8	58·9	54·3	50·8	48·7
Donor 3						
Fresh PBMC	27·0	18·5	10·3	0·9	0·5	0·5
PBMC+mock	23·5	17·5	9·5	2·1	0·3	0·5
PBMC+rotavirus	48·5	32·8	29·5	21·8	17·5	14·4

*Freshly isolated PBMC, PBMC cultured with control antigen made of mock-infected MA104 cells and PBMC cultured with rotavirus for 7 days were tested for their ability to lyse K562 cells and LCL in ^{51}Cr-release assays at various effector:target cell ratios.

mechanisms of resistance to and recovery from various kinds of viral immunity (Santoli, Trinchieri & Lief, 1978; Kohl, Harmon & Tang, 1983; Yasukawa & Zarling, 1983; Froelich & Guiffaut, 1987), we examined the change in non-specific cytotoxic activity occurring after stimulation with rotavirus. The data in Table 2 show that PBMC from three individuals, following incubation with rotavirus, produced significantly greater lysis of NK-sensitive K562 cells than freshly separated PBMC and PBMC incubated with mock-infected MA104 antigen. Epstein–Barr virus-transformed LCL has been shown to be resistant to lysis by NK cells but susceptible to lysis mediated by IL-2-stimulated lymphocytes, termed LAK cells (Zarling *et al.*, 1981; Yasukawa & Zarling, 1983). The results in Table 2 show that fresh PBMC and mock-stimulated PBMC were not capable of lysing LCL, whereas PBMC incubated with rotavirus exerted high cytotoxicity against LCL. Therefore, it is suggested that PBMC exposed to rotavirus have augmented non-specific cytotoxicity, such as NK activity and LAK activity.

Table 3. Reduced cytotoxic activity of effectors for fresh PBMC and rotavirus-activated killer cells by treatment with monoclonal antibody and complement

Treatment	% specific ^{51}Cr release* K562 40:1	20:1	10:1	MJ-LCL 40:1	20:1	10:1
Donor 1						
Fresh PBMC						
C alone	41·2	32·5	17·8	5·2	3·1	1·3
OKT8+C	37·5	30·1	15·8	4·6	3·5	1·0
Leu11b+C	2·2	1·6	1·0	0·2	0·6	0·7
NKH1A+C	2·5	1·1	1·2	−0·2	−0·3	0·8
PBMC+rotavirus						
C alone	71·7	62·6	52·2	70·4	60·3	57·4
OKT8+C	67·7	60·3	51·0	70·3	58·0	54·5
Leu11b+C	58·1	48·4	41·2	56·6	45·8	35·0
NKH1A+C	37·0	24·4	12·7	32·5	26·7	13·3
Donor 2						
Fresh PBMC						
C alone	30·5	19·8	10·2	3·6	3·0	2·2
OKT8+C	27·3	15·4	7·3	3·0	1·2	0·5
Leu11b+C	1·1	0·8	0·8	0·3	0·1	0·3
NKH1A+C	1·2	1·0	0·8	0·2	−0·3	−0·2
PBMC+rotavirus						
C alone	59·6	51·9	36·4	45·0	39·8	29·3
OKT8+C	56·7	52·8	37·0	46·8	40·8	33·5
Leu11b+C	46·6	37·5	22·1	39·8	22·1	16·3
NKH1A+C	20·0	15·5	7·8	21·5	15·8	9·2

*Freshly isolated PBMC and rotavirus-stimulated PBMC were treated with monoclonal antibodies and complement as detailed in Materials and Methods. Then they were tested for their ability to lyse K562 cells and LCL in ^{51}Cr-release assays at various effector:target cell ratios.

Characteristics of effectors for rotavirus-activated killer cells

We next determined the phenotype of lymphocytes exerting non-specific cytotoxicity after stimulation with rotavirus. The monoclonal antibodies Leu11b and NKH1A are considered to react with the vast majority of lymphocytes mediating NK activity (Hercend *et al.*, 1985; Itoh *et al.*, 1985b), and therefore we used these antibodies in addition to OKT8 (anti-CD8), considered to react mainly with HLA class I-restricted cytotoxic T lymphocytes (CTL) (Zarling & Kung, 1980), for phenotypic analysis of rotavirus-activated cytotoxic effectors. The data shown in Table 3 indicate that the phenotypes of freshly separated lymphocytes mediating NK activity are Leu11b$^+$ and NKH1A$^+$, as previously reported (Hercend *et al.*, 1985; Itoh *et al.*, 1985b). However, the treatment of rotavirus-activated lymphocytes with Leu11b or NKH1A and complement only partially reduced their cytotoxic activity against K562 and LCL. These results suggest that multiple subsets of lymphocytes

Table 4. Reduced cytotoxic activity of precursors for rotavirus-activated killer cells by treatment with monoclonal antibody and complement

	% specific ^{51}Cr release*					
	K562			MJ-LCL		
Treatment	40:1	20:1	10:1	40:1	20:1	10:1
Donor 1						
C alone	77·6	76·3	66·9	64·5	59·3	45·9
OKT8+C	73·4	64·8	47·7	58·1	55·3	42·6
Leu11b+C	12·3	10·0	7·3	16·1	9·0	4·3
NKH1A+C	31·5	16·7	8·7	27·0	18·7	14·5
Donor 2						
C alone	49·8	36·6	18·3	39·7	25·0	11·4
OKT8+C	53·0	36·4	21·4	41·0	26·7	12·0
Leu11b+C	5·8	0·9	0·1	2·3	0·4	−2·1
NKH1A+C	18·1	11·2	5·3	9·2	4·1	1·3

* PBMC treated with monoclonal antibodies and complement were cultured with stimulation by rotavirus as detailed in Materials and Methods. Then they were tested for their ability to lyse K562 cells and LCL in ^{51}Cr-release assays at various effector:target cell ratios.

Table 5. IL-2 and IFN-γ production by rotavirus-stimulated PBMC

		IL-2 production (ct/min)*		IFN-γ production (U/ml)†	
		Stimulation with rotavirus			
Donor	Incubation time (days)	+	−	+	−
1	2	5493	602	47	<10
	4	2575	832	273	<10
	6	2342	799	485	<10
2	2	8368	539	104	<10
	4	6523	821	203	<10
	6	3991	803	493	<10
3	2	8321	902	131	<10
	4	5479	1035	305	<10
	6	3284	874	498	<10

* The assays for IL-2 were performed by measurement of ^{3}H-TdR incorporation into IL-2-dependent CTLL cells as detailed in Materials and Methods. The results are mean ct/min for triplicate wells.

† The assays for IFN-γ were performed by radioimmunoassay using a monoclonal antibody to IFN-γ.

become cytotoxic in response to stimulation with rotavirus. This characteristic is similar to that of LAK cells, which consist of multiple lymphocyte subpopulations mediating cytotoxic activity in response to IL-2. Treatment with OKT8 and complement had little effect on cytotoxicity, indicating that the augmentation of cytotoxic activity by rotavirus was not mediated by antigen-specific CTL.

Characteristics of precursors for rotavirus-activated killer cells

Fresh PBMC were treated with each of the monoclonal antibodies OKT8, Leu11b and NKH1A plus complement and tested for their ability to lyse K562 cells and LCL after 7 days of incubation with rotavirus. As shown in Table 4, the degree of cytotoxic activity of $CD8^-$ cells against K562 and LCL was almost the same as that of whole PBMC, whereas pretreatment of PBMC with Leu11b or NKH1A and complement resulted in reduction of cytotoxicity against both K562 cells and LCL after stimulation with rotavirus. These results suggest that precursors for rotavirus-activated killer cells are largely present in the $CD16^+$ and $NKH1A^+$ populations, the former being predominant.

Production of IL-2 and IFN-γ

It is known that NK activity is augmented by various lymphokines, such as IL-2 and IFN (Henney *et al.*, 1981; Itoh *et al.*, 1985a; Ortaldo, Mason & Overton 1986), and that some NK-resistant cells are susceptible to lysis by lymphocytes incubated with IL-2, the latter being termed LAK cells (Grimm *et al.*, 1982). Thus we speculated that the non-specific cytotoxicity of rotavirus-activated cells may be augmented by lymphokines produced by rotavirus-reactive helper T cells. To clarify this possibility, we examined activities of IL-2 and IFN-γ in culture supernatants of lymphocytes stimulated with rotavirus. The data, presented in Table 5, indicated that both IL-2 and IFN-γ were present in culture supernatants of rotavirus-stimulated lymphocytes but not in those of unstimulated lymphocytes. Thus, it is suggested that stimulation of lymphocytes with rotavirus generates the specific production of IL-2 and IFN-γ, and that such lymphokines augment the non-specific cytotoxic activity of $CD16^+$ and/or $NKH1A^+$ lymphocytes. Kinetic studies of IL-2 and IFN-γ production showed that the peak activity of IL-2 occurred at 2 days, whereas IFN-γ activity continued to rise throughout the 6-day experimental period.

DISCUSSION

The present series of experiments revealed the following findings: PBMC of healthy adults proliferate, producing IL-2 and IFN-γ, in response to stimulation with rotavirus; $CD4^+$ T cells, which are thought to be helper T cells, are predominantly activated by rotavirus; and stimulation with rotavirus augments the non-specific cytotoxic activity mediated by $CD16^+$ and $NKH1A^+$ cells.

Recently, Totterdell *et al.* (1988) found that PBMC of healthy individuals, but not lymphocytes isolated from cord blood, proliferate in response to stimulation with rotavirus. Their observation and our present study suggest that rotavirus-specific memory T cells circulate in peripheral blood and may also exist in the intestinal mucosa, which is the primary region of rotavirus infection.

We found that human PBMC also proliferated in response to bovine rotavirus even though the response was lower than that produced by human rotavirus. Although it has been reported that the bovine NCDV strain is serologically distinct from any of the human rotavirus strains (Hoshino *et al.*, 1984), infants and young children were found to be protected against challenge with vilulent human rotavirus after oral inoculation with the bovine strain (Vesikari *et al.*, 1985). Furthermore, Offit & Dudzik (1988) have recently reported that rotavirus-specific murine CTL cross-react with target cells infected with different rotavirus serotypes. Taken together with this finding, our present data suggest that cell-mediated immunity may mediate

heterotypic protection against rotavirus infection. However, in this study, human PBMC responded to bovine rotavirus only at a high concentration of viral antigen. This result suggests that the frequency of T cells cross-reactive with bovine rotavirus is relatively lower than that of human rotavirus-specific T cells. Clarification of the viral antigenic determinants recognized by virus-specific T cells is essentially important for the development of effective vaccines. Based on this concept, we are currently studying the antigen specificity of rotavirus-specific human T cell clones using various strains of rotavirus and recombinant viruses.

A proliferative response of PBMC was observed for infectious rotavirus as well as for u.v.-inactivated virus. This finding suggests that the infectiousness of rotavirus does not essentially influence the cell-mediated immune response to rotavirus. However, Morrison, Braciale & Braciale (1988) reported that influenza virus-specific MHC class I-restricted $CD8^+$ CTL were preferentially induced by stimulation with infectious virus, whereas u.v.-inactivated virus preferentially activated MHC class II-restricted $CD4^+$ CTL. Their finding suggests that infectious and non-infectious viruses are differentially processed by antigen-presenting cells. Therefore, further studies are needed to examine whether differences exist between the immune responses to infectious and inactivated rotaviruses, since it is important to clarify this point for vaccine design.

The intestinal mucosa is a major host barrier to foreign antigen entry. Recent phenotypic and functional studies have revealed that intestinal mucosa is rich in CTL and NK cells (Klein & Kagnoff, 1984; Ernst *et al.*, 1986; Bonneville *et al.*, 1988; Goodman & Lefrancois, 1988). These findings suggest that cytotoxicity mediated by CTL and NK cells is an important defence mechanism against viral infection in the intestine. In the present study, we found that non-specific cytotoxic activity was augmented by culturing PBMC with rotavirus. In addition, studies of lymphokine secretion revealed that IL-2 and IFN-γ, which are both known to be mediators for augmentation of non-specific cytotoxic activity (Henney *et al.*, 1981; Itoh *et al.*, 1985b; Ortaldo *et al.*, 1986), were detected in culture supernatants of PBMC stimulated with rotavirus. Based on these findings, we speculated that the augmented NK activity and generation of cytotoxicity against NK-resistant cells were both mediated by lymphokines, such as IL-2 and IFN-γ, produced by rotavirus-reactive $CD4^+$ helper T cells. It has been reported that various kinds of virus-infected cells are susceptible to lysis by NK and LAK cells (Santoli *et al.*, 1978; Kohl *et al.*, 1983; Yasukawa & Zarling, 1983; Froelich & Guiffaut, 1987), and that cytotoxic effector cells can directly inhibit viral replication in target cells (Fitzgerald, Mendelsohn & Lopez, 1985; Yasukawa & Kobayashi, 1985). Therefore, it seems likely that one of the defence mechanisms of rotavirus infection at the intestinal mucosa is cell-mediated immunity.

We have first demonstrated the circuit of cell-mediated immune response to rotavirus in humans. In addition to humoral immunity, such cell-mediated immunity might play an important role in protection and recovery from rotavirus infection.

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