The Effect of Adoptive Transfer of Mononuclear Leukocytes from an Adult Donor on Spontaneous Cell-mediated Cytotoxicity and Resistance to Transmissible Gastroenteritis in Neonatal Piglets

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ABSTRACT

The purpose of this study was to attempt to establish spontaneous cell-mediated cytotoxicity effector activity in the intraepithelial lymphocytes of neonatal piglets by adoptive transfer of mononuclear leukocytes from an adult donor and to determine the effect of transfer on the resistance of piglets to transmissible gastroenteritis. Cytotoxicity was determined by a chromium release assay using PK-15 cells persistently infected with transmissible gastroenteritis virus as targets. The experimental animals were inbred miniature pigs, in which a high degree of uniformity in lymphocyte defined histocompatibility complex antigens was demonstrated by the mixed lymphocyte reaction. Adoptive transfer of 8 x 10^7-4 x 10^8 adult pig leukocytes established effector activity in eight recipient piglets, and leukocytes labelled with fluorescein isothiocyanate homed to the epithelium of the small intestine. When four recipients of 5 x 10^8 adult leukocytes were challenged with transmissible gastroenteritis virus, the onset of diarrhea was delayed for 24 h and the diarrhea was usually milder than in four untreated control piglets. It was concluded that the adoptive transfer of leukocytes with spontaneous cell-mediated cytotoxicity effector activity, which homed to the small intestinal epithelium, may have contributed to an increased resistance to transmissible gastroenteritis.

Key words: Coronaviridae, transmissible gastroenteritis, spontaneous cell-mediated cytotoxicity.

RÉSUMÉ

Cette expérience visait à déterminer l'activité réelle de la cytotoxicité spontanée à médiation cellulaire des lymphocytes intraépithéliaux des porcelets nouveau-nés, au moyen de la transfusion de mononucléaires d'un jeune verrat adulte; elle consistait aussi à rechercher l'effet d'une telle transfusion sur la résistance des porcelets à la gastro-entérite transmissible. Les auteurs déterminèrent la cytotoxicité au moyen d'une épreuve de relâchement du chrome qui utilisait comme cible des cellules PK-15 constamment infectées par le virus de la gastro-entérite transmissible. Les animaux d'expérience étaient des porcs nains consanguins, qui possédaient un degré élevé d'uniformité dans l'histocompatibilité des complexes antigéniques de leurs lymphocytes, comme le démontra la réaction d'un mélange de ces lymphocytes. La transfusion de 8 x 10^7 à 4 x 10^8 leucocytes du verrat précité à huit porcelets se traduisit par une activité effectrice et les leucocytes marqués à l'isothiocyanate de fluorescéine se localisèrent dans l'épithélium de l'intestin grêle des porcelets. L'infection de défi de quatre porcelets qui avaient recu 5 x 10^8 leucoytes de leur congénère adulte, avec le virus de la gastro-entérite transmissible, retarda d'une journée le début de la diarrhée qui s'avéra ordinairement moins profuse que chez les témoins. Les auteurs conclurent que la transfusion de leucocytes pourvus d'une activité effectrice de la cytotoxicité spontanée à médiation cellulaire qui se localisèrent dans l'épithélium de l'intestin grêle des porcelets expérimentaux pourrait avoir accru leur résistance à la gastro-entérite transmissible.

Mots clés: *Coronaviridae*, gastro-entérite transmissible, cytotoxicité spontanée à médiation cellulaire.

INTRODUCTION

The inability of peripheral blood lymphocytes (PBL) from newborn piglets to serve as effector cells in spontaneous cell-mediated cytotoxicity (SCMC) against human myeloid leukemia K-562 target cells has been established (1,2). We extended these studies by demonstrating that PBL from newborn piglets also failed to show activity against target cells (PK-15-TGE) infected with transmissible gastroenteritis virus (TGEV) and we demonstrated a similar lack of SCMC activity against PK-15-TGE targets among intraepithelial lymphocytes (IEL) from newborn piglets (3). Peripheral blood lymphocytes and IEL from older piglets and adult swine mediated high levels of SCMC against PK-15-TGE cells (3,4). K cell activities in antibody-dependent cell-mediated cytotoxicity (ADCC) were also lacking in the PBL and IEL from newborn piglets (3). In our earlier paper (3), we

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postulated that the lack of these activities in newborn piglets might contribute to their high susceptibility to TGE.

The objectives of the studies described in the present paper were to attempt to establish SCMC in neonatal piglets by the adoptive transfer of mononuclear leukocytes from an adult donor and to determine the effect of such transfer on the resistance of the neonate to challenge with TGEV. Commercially available inbred miniature pigs were used as donor and recipients. Since no data were available relating to the degree of histocompatibility among the animals, this was investigated by conducting mixed lymphocyte reactions between donor and recipients. Tests were also conducted to determine whether the adoptively transferred leukocytes gained access to the epithelium of the small intestine and conveyed SCMC activity to the recipients.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Miniature pigs were purchased from Vita Vet Labs, Inc., Marion, Indiana. Two pregnant sows and one young adult male were used. The sows produced litters of 11 and eight piglets which served as recipients for leukocyte transfer and the adult male was used as the leukocyte donor. The pigs were negative for TGE virus neutralizing antibodies as determined by a microtitre virus neutralization test against 50 median tissue culture infectious doses of TGEV. The pigs were held in strict isolation and fed Purina dog food (1% of body weight daily). The piglets were allowed to nurse their dams. Conventional piglets were obtained from a specific pathogen-free herd with no history of TGE and lacking TGE virus neutralizing antibodies.

ISOLATION OF MONONUCLEAR LEUKOCYTES FOR ADOPTIVE TRANSFER

Venous blood from the adult male pig was collected into heparin (10 units per mL of blood) as required. The heparinized blood was incubated for 1 h at 37°C on a rotating mixer with 0.1 g of carbonyl iron powder per mL. After the phagocytic cells had been removed with a strong magnet, the blood was diluted with an equal volume of cold Hanks' balanced salt solution (HBSS), layered in 20 mL volumes over 8 mL of Ficoll-Hypaque (Pharmacia, Dorval, Quebec) in siliconized Corex tubes and centrifuged at 400 g for 30 min. The mononuclear leukocytes recovered from the interface were washed three times and resuspended in RPMI-1640 medium (Grand Island Biological Co., Grand Island, New York) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 6 mM Hepes buffer (Calbiochem-Behring Corp., La Jolla, California), 2 mM L-glutamine and 50 μg per mL of gentamycin (complete RPMI-1640 medium). The cell suspension was kept on ice until used. Since we have found that carbonyl iron treatment does not remove all monocytes, we refer to the cell suspensions prepared as described above as mononuclear leukocytes rather than lymphocytes. The PBL used as effector cells in the SCMC assay described below were subjected to the additional step of plastic adherence for the removal of monocytes (3).

SCMC CHROMIUM RELEASE ASSAY

Peripheral blood lymphocytes and IEL were isolated from the piglets as described previously (3), and used as effector cells in the SCMC assay (4). The target cells were PK-15 cells persistently infected with TGEV (PK-15-TGE cells). An effector/target cell ratio of 50:1 and an incubation period of 16 h were used. Appropriate controls were included as before and all determinations were made in triplicate except for the controls for total and spontaneous ^{51}Cr release, which were made in sextuplicate. The results were expressed as % specific ^{51}Cr release computed at 100 x [(mean cpm effector + target cells)–(mean cpm with target cells only)/(total releasable cpm)–(mean cpm target cells only)]. Statistical analysis of differences between mean values were performed by the paired t-test.

ONE WAY MIXED LYMPHOCYTE REACTION

This test was performed with stimulator cells from the donor pig and responder cells from 11 newborn miniature piglets and from eight conventional newborn piglets for comparison. Stimulator and responder mononuclear leukocytes were isolated as described above. Stimulator cells were resuspended at 2 x 10^6 cells per mL in RPMI-1640 medium containing 25 μg per mL of mitomycin C and incubated for 30 min in a 37°C water bath with occasional shaking. The cells were then washed three times in calcium and magnesium free phosphate buffered saline (PBS) with centrifugation for 10 min at 4°C at 300 g and resuspended in complete RPMI-1640 medium at 3 x 10^6 cells per mL. Responder leukocytes were suspended in complete RPMI-1640 medium at 1.5 x 10^6 cells per mL. The test was carried out in Linbro 96-well round-bottom microtest plates in sextuplicate. One hundred microlitres of the stimulator cell suspension and an equal volume of responder cell suspension were mixed in each well. The controls contained stimulator or responder cells only in 200 μL of complete RPMI-1640 medium. The plates were incubated for five days at 37°C in 5% CO_2. The cells were then pulsed with 2 μCi of (^{3}H) thymidine in 50 μL of complete RPMI-1640 medium per well and incubation was continued for an additional 24 h. The cells were harvested on filter discs with an automatic cell harvester, air dried and transferred to vials containing 4.0 mL of scintillation cocktail (3.8 L scintillized toluene, 10 g 2,5-diphenyloxazole, 237.5 mg p-bis [2(5-phenyloxazolyl)-benzene]). The radioactivity was measured in a Tri-Carb 460 liquid scintillation counter (Packard Instrument Company Inc., Downers Grove, Illinois). Stimulation indices (SI) were calculated for each piglet from the counts of stimulated and unstimulated responder cells.

LABELLING OF LEUKOCYTES WITH FLUORESCEIN ISOTHIOCYANATE

The procedure was based on the technique used in mice (5,6). The labelled cells were resuspended to the required concentration in complete RPMI-1640 medium and kept on ice until used.

LEUKOCYTE TRANSFER AND SAMPLE COLLECTION

A litter of 11 inbred miniature piglets was used to study the distribu-

tion of transferred cells and the appearance of SCMC activity, in the recipients of SCMC competent mononuclear leukocytes from the adult donor. Unlabelled or FITC-labelled leukocytes were warmed briefly in a 37°C water bath and administered to two days old piglets, narcotized with halothane, by intracardiac injection. Three piglets received 8 x 10^7 unlabelled cells, two received 4 x 10^8 unlabelled cells and three received 4 x 10^8 labelled cells. Three piglets were left as untreated controls. In addition, two conventional outbred piglets of the same age received 4.0 mL of FITC labelling solution in order to determine whether free label would cause tissue fluorescence. The piglets were killed, 18, 48, 60 or 90 h after transfer of leukocytes, by an intravenous overdose of sodium pentobarbitone. Peripheral blood lymphocytes and IEL were isolated and tested for effector activity in the SCMC assay as described above. Cryostat sections were prepared from the small intestine, mesenteric lymph nodes, thymus, spleen, popliteal lymph node and liver and examined under a fluorescence microscope.

CHALLENGE WITH TRANSMISSIBLE GASTROENTERITIS VIRUS

A litter of miniature piglets was divided into two groups of four piglets. Each piglet in the experimental group received 5 x 10^8 unlabelled mononuclear leukocytes from the adult donor, prepared as described above, by intracardiac injection at two days of age. The controls received complete RPMI-1640 medium only. Forty hours later each piglet was dosed orally with the virulent Purdue strain (7) of TGE virus. The virus inoculum consisted of 1.0 mL of a 1:100 dilution in PBS of a 20% suspension of small intestinal mucosa and contents, prepared as described (8), from an SPF piglet that had been infected with Purdue virus 48 h previously. The piglets were observed clinically, and the consistency of the feces was noted individually after anal irritation.

RESULTS

ONE WAY MIXED LYMPHOCYTE REACTION

The SI obtained ranged from 0.9 to 8.6 (Table I). Statistical analysis showed that SI of 1.6 or lower were not significant at the $P \leqslant 0.05$ level. Eight of the eleven miniature piglets failed to respond in the reaction and the remainder responded with SI lower than 3.0. In contrast, five of the outbred conventional piglets responded strongly, with SI ranging from 6.9 to 8.6, two responded weakly and one failed to respond.

TABLE I. Results of One Way Mixed Lymphocyte Reaction[a]

Piglet No.	Mean[b] 2 min Counts ±SD: Unstimulated Lymphocytes	Mean[b] 2 min Counts ±SD: Stimulated Lymphocytes	Stimulation Index
Inbred			
1	805 ± 251	1240 ± 484	1.5[c]
2	808 ± 242	2101 ± 421	2.6
3	843 ± 238	1263 ± 304	1.5[c]
4	810 ± 269	985 ± 347	1.2[c]
5	795 ± 208	785 ± 198	0.9[c]
6	783 ± 256	1609 ± 396	2.0
7	816 ± 307	1316 ± 557	1.6[c]
8	828 ± 211	1240 ± 264	1.5[c]
9	799 ± 226	944 ± 437	1.2[c]
10	825 ± 234	2356 ± 654	2.9
11	831 ± 238	990 ± 170	1.2[c]
Outbred			
1	1070 ± 362	7833 ± 1904	6.9
2	1102 ± 291	7934 ± 1886	7.2
3	1028 ± 287	2467 ± 596	2.4
4	1006 ± 314	8651 ± 2082	8.6
5	1093 ± 263	1748 ± 408	1.6[c]
6	1088 ± 312	8595 ± 2062	7.0
7	1006 ± 406	2213 ± 526	2.2
8	1035 ± 589	8590 ± 2085	8.3

[a]The stimulator cells were obtained from the adult miniature pig and the responder cells were from inbred miniature piglets or outbred conventional piglets

[b]Mean values are based on sextuplicate estimations

[c]Stimulation indices not significant at the $p \leqslant 0.05$ level

SCMC ACTIVITY AND FATE OF DONOR LEUKOCYTES IN RECIPIENT PIGLETS

The specific SCMC ^{51}Cr release mediated by the donor's leukocytes was 35%. Significant SCMC ^{51}Cr release was not detected in any of the piglets which were tested before transfer, nor in the PBL or IEL of the untreated piglets (Table II). Spontaneous cell-mediated cytotoxicity activity was detected in the PBL or IEL of all the piglets which received leukocytes by adoptive transfer. The activities in the IEL were always higher than in PBL. High levels of SCMC activity could still be detected in IEL 90 h after transfer, but the activity in PBL declined rapidly and was undetectable 60 h after transfer. Further, it was observed that the piglets transfused with FITC labelled cells had similar levels of SCMC activity to those transfused with unlabelled cells.

Fluorescence microscopy of the cryostat sections showed no discrete cellular fluorescence in any of the controls, including those injected with free FITC labelling solution. The occurrence of fluorescence in the small intestinal epithelium following the injec-

TABLE II. SCMC Activities and Fluorescence in Recipient Piglets After the Adoptive Transfer of Mononuclear Leukocytes from an Adult Donor

Piglet Number	Number of Cells Transferred	Euthanasia (h after transfer)	% Specific SCMC^{51} Cr Release: Before Transfer PBL[a]	After Transfer PBL	After Transfer IEL[a]	Fluorescence[b]
1	8 x 10^7	18	NT	9.0	26.1	0
2	4 x 10^8	18	-2.5[d]	16.9	67.0	0
3	[c]4 x 10^8	18	-1.7[d]	21.7	58.9	+++
4	0	18	NT	-0.8[d]	1.3[d]	0
5	8 x 10^7	48	NT	6.5	24.8	0
6	[c]4 x 10^8	48	-2.6[d]	9.6	69.3	++++
7	0	48	NT	-1.2[d]	0.9[d]	NT
8	8 x 10^7	60	-2.8[d]	-0.2[d]	18.9	NT
9	4 x 10^8	90	NT	1.6[d]	66.6	0
10	[c]4 x 10^8	90	NT	3.1[d]	68.8	++++
11	0	90	-0.5[d]	0.6[d]	1.5[d]	0

[a]PBL = peripheral blood lymphocytes; IEL = intraepithelial lymphocytes

[b]Extent of fluorescence in the small intestinal epithelium graded 0 to ++++

[c]Leukocytes labelled with FITC

[d]Not significant

NT = not tested

TABLE III. Response of Piglets to Challenge with TGE Virus

Piglet Number	Number of Leukocytes Transferred (10^8)	Occurrence of Diarrhea after Challenge									
		Hours				Days					
		18	24	36	42	3	4	5	6	7	8
1	5	–	–	–	+	+	+	+	–	–	–
2	5	–	–	–	++	[b]					
3	5	–	–	–	+	+	+	+	+	–	–
4	5	–	–	–	+	+	+	+	+	–	–
5	0	++	++	++	++	++	[b]				
6	0	+	++	++	++	++	++	++	+	–	–
7	0	++	++	++	++	++	[b]				
8	0	+	++	++	++	++	++	++	+	–	–

[a]Diarrhea graded as – (none), + (mild) or ++ (profuse, watery)

[b]Piglet died

tion of labelled cells is recorded in Table II. Epithelial fluorescence was detected 18 h posttransfer. At this time, many fluorescent cells were also seen in blood vessels, loosely organized Peyer's patches and in the lamina propria. At 48 and 90 h after transfer, large numbers of fluorescent cells were still seen in the epithelium, but the lamina propria was virtually negative and few fluorescent cells were present in Peyer's patches. Fluorescent cells were also detected in the following tissues, in order of decreasing frequency: mesenteric lymph node, spleen, popliteal lymph node, thymus and liver.

RESPONSE TO CHALLENGE WITH TRANSMISSIBLE GASTROENTERITIS VIRUS

The results are summarized in Table III. The control piglets (numbers 5-8) developed diarrhea 18 h after challenge and their feces soon became watery. The onset of diarrhea in the piglets that had received mononuclear leukocytes (numbers 1-4) was delayed until 42 h after challenge and the diarrhea was generally milder than in the control piglets. Two control piglets and one leukocyte recipient died of TGE. The remaining piglets recovered from the infection on the seventh day after challenge.

DISCUSSION

The miniature piglets used in this study were stated by the supplier to have been inbred for 46 generations. The results of the mixed lymphocyte reaction, in which there was minimal stimulation of the responders' lymphocytes by the donor's lymphocytes, demonstrated a high level of uniformity in lymphocyte defined histocompatibility antigens. This was an important finding since others (9) have shown that allogeneic stimulation of human PBL in the mixed lymphocyte reaction can enhance NK and K cell activities, as well as generating cytotoxic T lymphocytes. However, since in the human study substantial stimulation was not detected until three days after the mixed lymphocyte reaction had been started, it is unlikely that the appearance of SCMC activity 18 h after leukocyte transfer resulted from allogeneic stimulation of the recipients' lymphocytes.

The most widely used techniques for cell labelling utilize radioactive markers, but the labelled cells cannot be individually identified while viable and most radioisotopes fail to label all cell types equally. The recently introduced technique of fluorescent labelling (5,6) avoids these problems. Murine lymphocytes can be labelled without loss of viability and they retain the ability to mount a graft versus host reaction (6). Furthermore, labelling does not alter normal lymphocyte migration or the expression of surface antigens and the labelled cells can be demonstrated in the tissues of recipient animals for at least 11 days after intravenous injection. Another recent paper describes the successful labelling, with excellent cell survival, of porcine lymphocytes with FITC (10). In our experiments, direct labelling of leukocytes with FITC did not interfere with SCMC activity in the recipients and it proved to be an effective tracer of the transferred cells.

Newborn piglets have fewer IEL than adults (11) and IEL from newborn piglets lack SCMC and ADCC effector activities for virus-infected target cells (3). It is not clear whether the small number of IEL in the newborn is caused by a lack of suitable cells in the circulation, or by failure of the epithelium to incorporate the lymphocytes. The existence of a factor attracting IEL within murine intestine has been postulated (12). The SCMC activity of the IEL which we isolated from the recipient piglets was higher than that of the donor's leukocytes. Taking into account the dilution of the donor's cells in the recipients' tissues, it seems reasonable to conclude that the SCMC effector cells homed selectively to the small intestinal epithelium. This view is supported by the relatively rapid clearance of SCMC activity from the recipients' blood, by the low numbers of fluorescent cells in the liver, which is known to be involved in the elimination of dead cells from the circulation, and by the incorporation of large numbers of labelled cells into the recipients' intestinal epithelium. These findings suggest that limited availability of the appropriate lymphocyte sub-population may be responsible for the low IEL content in newborn piglets. The detection of SCMC activity in IEL from transfused piglets simultaneously with the appearance of donor's cells in the intestinal epithelium and the persistence of this activity for at least 90 h after transfusion, indicate the lack of a SCMC inhibiting factor in newborn piglets. In an earlier paper (3) we suggested that the lack of cytotoxic lymphocytes in the newborn might be related to an inhibitory effect of high levels of corticosteroids, estrogens and prostaglandins at this time, but in the light of our present findings, late maturation of the SCMC effector cells seems a more likely explanation.

When the recipients of the adult donor's leukocytes were challenged with TGEV, the onset of diarrhea was delayed by 24 h compared with the control piglets. During this period the transfused piglets would be exposed to high levels of virus excreted by their untreated litter mates. Although the acquired resistance factor was then overcome and one of the treated piglets died, the diarrhea in the remaining treated animals was milder than in the untreated controls, two of which died. It is believed that the adoptive transfer of SCMC effector

cells, which homed to the small intestinal epithelium may have contributed to the increased resistance of these piglets to TGE. The adoptive transfer of K cells may have contributed to the mildness of the diarrhea in the recipients, since we have previously detected antibody participating in ADCC in the intestinal washing of piglets with TGE as early as three days postinfection, whereas virus neutralizing antibody was not detected until two days later (13). Confirmation of these findings is required since only small numbers of animals were used in this experiment due to the high cost and limited availability of inbred piglets suitable for adoptive transfer experiments. However, the results suggest that it might be beneficial to attempt stimulation of NK cell activity immediately after birth and experiments with this objective are currently in progress.

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