Immunogenicity of a plant-derived edible rotavirus subunit vaccine transformed over fifty generations

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Abstract

Major efforts have been put forth for the development of effective rotavirus vaccines including transgenic plant vaccines. Previous studies have reported that rotavirus VP7 maintains its neutralizing immunity when it is transformed into the potato genome. The present study was aimed at investigating the hereditary stability of VP7-transformed potatoes over fifty generations. The VP7 gene was stably transcribed and expressed in potato cells as detected by RT-PCR and Western blotting. Humeral and mucosal responses were successfully induced in BALB/c mice fed with the fiftieth generation transformed potato tubers. There were no significant differences in serum IgG and fecal IgA between the mice fed with the first and fiftieth generation potatoes (P >0.05). Profiles of cytokines such as IFN-gamma, IL-2, IL-4, IL-5 and TGF-beta in immunized mice showed a naive T-cells bias to Th1 and Th3 polarization. Moreover, specific CTL responses were also detected in C57BL/6 mice fed with transformed potatoes. This research represents a significant step towards the development of rotavirus vaccines derived from a transgenic plant that can be obtained by long-term and large-scale vegetative reproduction. To our knowledge, this is the first finding regarding vaccines derived from plants that can be propagated for many generations.

Keywords: Transgenic plant; Rotavirus VP7; Edible vaccine; Cytokine

Introduction

Rotavirus is the most important cause of viral gastroenteritis in young children and animals in the world (Parashar et al., 2003). Most children have been infected with rotavirus at least once by the age of 3, approximately six million children die each year from diarrhea caused by a rotavirus infection in developing countries (O’Ryan et al., 1994). Therefore, it is imperative to develop safe and effective vaccines to fight against rotavirus infections (Velazquez et al., 1996). The outer capsid of rotavirus comprises a major glycoprotein VP7 and a minor and protease-sensitive protein VP4. VP4 and VP7 can induce neutralizing antibodies; therefore, they can be used as targets for vaccine development (Buesa et al., 1999).

Major efforts have been devoted to the development of effective rotavirus vaccines for humans and animals because the discovery of the virus (Bernstein et al., 1999; Madore et al., 1992). Live attenuated rotaviruses, rotavirus reassortants, virus-like particles composed of rotavirus proteins VP4, VP6 and VP7, DNA vaccines and rotavirus outer capsid protein (VP7 or VP6) expressed in plants have all been investigated (Yu and Langridge, 2001). Among them, transgenic plants expressing therapeutic proteins are ideal vehicles for producing and orally delivering protective antigens (Azad and Rojanasakul, 2006). It has been speculated that a plant’s cell wall delays the digestion of plant-derived antigens; therefore, more antigens are absorbed and presented to gut-associated lymphoid tissue (Ma et al., 2005). We and others have demonstrated that plant cells can express rotavirus antigenic proteins including VP7, in which the proteins have been shown to be antigenic and immunogenic (Yu and Langridge, 2001; Wu et al., 2003). In the present study, we investigated the stability of a full-length rotavirus VP7 gene in...
transgenic potatoes propagated for over fifty generations, in which the characteristics of a specific immune response in mice that were orally vaccinated with VP7-transformed potatoes were also studied.

Results

Transgenic potatoes can be propagated over fifty generations

We defined one generation as a two- to three-week period from when the seedlings grew up to when they were transferred to a fresh MS medium. We obtained over fifty generations of VP7 transgenic potato progenies within five years since 2001 when we obtained the first generation of transgenic potatoes. All of the seedlings maintained antibiotic characteristics (data not shown) by placing the fiftieth generation seedlings on an antibiotics selective MS medium (MS salts and vitamins, 20 g/l sucrose, 8.5 g/l agar, pH 5.6, 50 μg/ml kanamycin and 300 μg/ml cefotaxime). The stability of the transcription of a specific VP7 gene in the transformed potatoes was assessed by RT-PCR, as previously reported. In the serial progenies of potatoes transformed with VP7 gene, the amplification products were observed to correspond to the recombinant gene, whereas mock-transformed potatoes showed no transcription (Fig. 1A). The quality of VP7 protein was evaluated by Western blotting using MA104 cultured rotavirus Wa as the standard (Fig. 1B). VP7 protein expressed in house-cultured, evergreen tubers was subjected to Western blotting (Fig. 1C). The results indicated that VP7 protein could maintain its antigenic property, which were expressed in potatoes propagated from tubers or seedlings.

The difference in transformed potato progenies was assayed by the ELISA, in which the analysis showed that recombinant VP7 protein was expressed in the leaves at levels ranging from 3.6 to 4.0 μg/mg of the total soluble leaf protein (Fig. 2). Compared to our previous results (Wu et al., 2003) in which the amount of VP7 protein in tubers was equal to or slightly higher than it was in the leaves, it was here too that the amount of VP7 protein in the tubers was approximately 40 μg/g.

These results demonstrated that the VP7 gene could be inherited and stably expressed for more than fifty generations.

Induction of a humoral and mucosal immune response in orally immunized mice

BALB/c mice were immunized orally on days 0, 7, 14, 21 and 42 with 2 g of the fiftieth generation transgenic potato tubers. At different times, mouse sera and feces were collected and analyzed for the presence of specific anti-VP7 antibodies (Figs. 3A and B). Antibodies from experimentally immunized mice showed a strong response against VP7 as shown by the ELISA. After the initial inoculation, the animals

Fig. 1. Detection of VP7 gene transcription and expression level in different transgenic potato progenies. (A) Detection of VP7 gene transcription by RT-PCR. Total mRNA from different transgenic progenies; M, DNA molecular weight marker (Sangon, China); N, negative control (empty template); N’, negative control (mock transgenic potatoes); P, positive control (UV-inactivated RV from infected mammalian cell MA104 lysate); T1, T2, T3, T4, T5, T10, T20, T30, T40 and T50: the first, second, third, fourth, fifth, tenth, twentieth, thirtieth, fortieth and fiftieth generation VP7-transformed potato seedlings, respectively. (B) Western blots of the total soluble protein from T10, T20, T30, T40 and T50. (C) Western blots of total soluble protein from different progenies. G1, G2, G3 and G4: VP7-transformed potatoes planted in a greenhouse in the first, second, third and fourth year, respectively.
developed a specific immune response that was enhanced with each booster. No response was detected in the mice that were immunized with mock-transformed potatoes, even after repeated inoculations (Figs. 3A and B). Mouse feces, urine and saliva were also collected and detected as described above (Fig. 3B). No significant levels of IgG and IgA were observed in saliva or urine, whereas significant IgA levels were observed in the feces from the mice immunized with transgenic potato tubers (Fig. 3B). A neutralizing assay conducted later on showed that the sera or feces derived from mice fed with control transgenic plants had no neutralization activity against rotavirus Wa. Significant levels of neutralizing antibodies were, however, detected in the sera or feces collected from mice fed with transgenic plants or transgenic plants plus CTB (Table 1). These results confirm that transgenic plants can induce functionally active anti-rotavirus intestinal antibodies.

**Cytokine profiles**

Splenocyte proliferation is an important indicator for the existence of humoral and cellular immunity. The spleen was obtained from C57BL/6 mice two weeks after the final booster and was prepared into single cell suspensions. Subsequently, the spleen cells from each mouse were isolated and stimulated by using purified particles of UV-inactivated rotavirus Wa.

Cytokines released from the spleen cells stimulated by UV-inactivated rotavirus were measured in order to determine the primed pathway of immune responses. IFN-gamma, IL-2, IL-4, IL-5 and TGF-beta were detected in the spleen cell supernatants. Results showed in turn significant differences in the production of IFN-gamma among the groups \((P<0.05)\) (Fig. 4A), whereas there was a slight increase in the production of IL-2 in the mice fed with VP7-transformed potatoes or with VP7-transformed potatoes as well as CTB compared to those fed with mock-transformed potatoes (Fig. 4B). There were no significant differences in the production of IL-4 (Fig. 4C) and IL-5 among the groups (Fig. 4D). However, mice fed with transgenic potatoes displayed a two- to fourfold increase in the TGF-beta level (Fig. 4E) compared to the mice fed with mock transgenic potatoes.

**VP7-transformed potatoes induced a rotavirus-specific cytotoxic T-cell immune response**

To determine whether VP7-transformed potatoes stimulate a CTL response, splenocytes obtained from C57BL/6 mice were restimulated with UV-inactivated rotavirus Wa in vitro, and used as effector cells to lyse the target cells, namely, rotavirus Wa-infected P815 cells. A significant specific lysis of the target cells was observed in groups 5 and 6 (Fig. 5). The control effector cells from the mice immunized with mock-
transformed potatoes were not significantly lysed by the target cells (Fig. 5).

Discussion

In light of the global impact of the rotavirus disease in children, researchers have endeavored to develop vaccines that stimulate immune responses induced by a natural rotavirus infection; nevertheless, few vaccines were proven successful (Ward et al., 1995; Michael, 1999). The mechanisms by which natural rotavirus infection or immunization in turn elicits protection remain poorly understood (Ward, 2003). In general, it appears that serum IgA is a good predictor of clinical protection after natural infection, but there is no or only a poor correlation between serum IgA and vaccination with RRV-TV or other rotavirus vaccines (Ward, 2003; Heath et al., 1997; Madore et al., 1992). Interestingly, previous studies (Wu et al., 2003) along with the present study have indicated that mice fed with rotavirus VP7 transgenic plant were more likely to induce a mucosal immune response (especially intestinal IgA) than a humeral response (serum IgG). There were no significant

![Fig. 4. Cytokine profiles of spleen cells stimulated by recombinant VP7 protein. Single cell suspensions of spleen cells were stimulated by 20 μg/ml UV-inactivated RV Wa purified from cultured MA104 cells. After 48 or 96 h of culture, cell-free supernatants were collected and assayed for the presence of (A) IFN-gamma, (B) IL-2, (C) IL-4, (D) IL-5 and (E) TGF-beta using a sandwich-ELISA. Data were presented as mean±SEM. Asterisk: P<0.05.](image)

![Fig. 5. Rotavirus-specific CTL response to rotavirus Wa-infected P815 cells in the C57BL/6J (H-2Kb) mice fed with VP7 transgenic potatoes. Splenocytes were obtained two weeks after the last booster and used as the effector cells in the CTL assay. 51Cr-release assays were performed to assess the cytotoxicity against rotavirus Wa-infected P815 cells at various E:T ratios. In the mice fed with VP7 transgenic potatoes, or VP7 transgenic potatoes plus bacteria CTB, a significant cytotoxicity to P815 cells was observed in a dose-dependent manner. Approximately 50% specific lysis was observed at an effector to target (E:T) ratio of 100:1, a figure higher than that of the groups fed with mock transgenic potatoes. The data are representative of the three sets of experiments.](image)
differences in immune response between the mice fed with the only transgenic VP7 potatoes and transgenic VP7 potatoes plus bacteria recombinant protein CTB, which is a kind of mucosal adjuvant. The reasons explaining the absence of an immune response difference have involved the method used (CTB were dissolved in PBS and placed on the potato tuber slices before fed, we can therefore suppose that the CTB protein was digested by the gastrointestinal tract digestive juice after eating the tubers) or the transgenic plant itself has the mucosal adjuvant function because of its cell wall. A co-expressed antigen and CTB are ideal. Furthermore, there were no significant differences in serum IgG and fecal IgA between the mice fed with the first and fiftieth generation potatoes ($P > 0.05$) (data not shown). The mechanisms involved are still unknown. It may be of significance to further study the correlation of a mucosal immune response and different rotavirus vaccines such as transgenic plant vaccine and a live attenuated vaccine. To express a rotavirus antigen-encoding gene in plants is altogether a new strategy for vaccine development, in which the resulting vaccine could be an ideal mimic of a natural virus infection in terms of inducing an intestinal IgA response.

Recently, plants were used as recombinant bioreactors to express a number of proteins including drugs and potential vaccines (Rigano et al., 2006; Youm et al., 2005; Aziz et al., 2005; Pogrebnyak et al., 2005). Our results suggest that transgenic potatoes remained stable for fifty generations. In theory, plant transgenes mediated by Agrobacterium tumefaciens are inherited stably and persistently according to Mendelian law; nevertheless, to our knowledge no report is available regarding how long they can be steadily inherited. The present study addresses for the first time the stability of a foreign gene in transgenic potatoes, which can propagate for many generations.

We detected specific cytotoxic T lymphocytes (CTLs) in C57BL/6 mice fed with VP7 transgenic potatoes, suggesting cell immunity as another mechanism for an immune response induced by a transgenic plant antigen. In fact, the mechanisms for immunity against rotavirus infection are not completely understood. Some believe that antibodies, especially local immunity mediated by IgA, and induced by an active infection is necessary (Bernstein et al., 1986), whereas others emphasize that cell immunity such as specific CTLs plays an important role (Franco et al., 1993).

CTLs are thought to be important in controlling primary rotavirus infections and in the immune protection against reinfection (Franco and Greenberg, 1995; Franco et al., 1993). A role for CD8+ T-cells in protection from viral reinfection has also been recognized (Franco et al., 1993; McNeal et al., 1995). Experimental data on rotavirus infection in mice support the hypothesis that rotavirus-specific CTL may protect against disease. Rotavirus-specific CTLs are detected at the intestinal mucosal surface in mice within the first week of infection (Franco et al., 1997). CD8+ T-cells from rotavirus-immunized adult mice protect suckling mice against rotavirus gastroenteritis in the absence of rotavirus-specific neutralizing antibodies (Offit and Dudzik, 1989) and mediate the clearance of chronic rotavirus infection in SCID mice (Dharakul et al., 1990). Therefore, our results require further researches.

Th1 cells are known to produce IFN-gamma and IL-2, whereas Th2 cells produce IL-4 and IL-5, and Th3 cells primarily produce TGF-beta (Street and Mosmann, 1991); hence, the identification of the cytokine profiles of spleen cells helps to determine the route by which rotavirus-specific antibodies were produced. In addition, the development of the CD4+ Th cell subset is essential for eradicating infectious rotavirus. Moreover, Th2 cells generally promote the development of selected humoral immunity, including IgG expression (Amemiya et al., 2002). Previous studies indicated that the expression of IgA in mucus is higher than that of serum IgG, but little is known about the exact reasons (Wu et al., 2003). The data presented here suggest that the immune responses following oral immunization were provoked through the Th1- and Th3-type routes. Th3-type response also tends to be involved in this study in light of TGF-beta secretion. Th3 cells are a subset of unique cells that primarily produce TGF-beta and principally develop a mucosal immune response to orally administered antigens. Th3 also has been found to have the ability to induce IgA production (Sidonia et al., 2001). Because the secretory cytokine TGF-beta may promote IgA secretion, we hypothesize that the oral administration of a plant-derived rotavirus vaccine induces a stronger IgA response, possibly due to TGF-beta secretion. However, further researches are needed to clarify the processes involved.

In conclusion, our results suggest that the VP7 gene-transformed potatoes could be stably transcribed and expressed for fifty generations, and that the immune responses following oral immunization are mediated by Th1- and Th3-type routes.

Materials and methods

Production of VP7 transgenic potato tubers

The rotavirus antigen-encoding VP7 gene from a fecal isolate of human rotavirus from a Chinese child with diarrhea was inserted into plasmid pBI121 (Wu et al., 2003), in which the recombinant was mobilized into A. tumefaciens (LBA4404), and was then used to transform Solanum tuberosum L. cv. Taiwanhong. The transformed Taiwanhong was cured of A. tumefaciens and clonally propagated, in which Taiwanhong line 4 was selected because of its high level of VP7 expression. This line was then clonally propagated. Every two or three weeks the seedling stem apex was cut and transferred to a fresh MS medium. For every ten generations, some potato seedlings were planted in soil to produce potato tubers and then collected for further detection and the others were propagated on MS medium. From 2001 to 2005, VP7-transformed potatoes were propagated for over fifty generations.

Western blot analysis of a VP7 protein expression in transgenic potatoes

Five micrograms of total soluble protein per sample were added to 6 μl 6× SDS gel loading buffer (300 mM Tris–HCl,
pH 6.8, 600 mM dithiothreitol, 12% SDS, 0.6% bromophenol blue, 60% glycerol), boiled for 10 min and placed on ice. Purified rotavirus particles were used as the positive control. The samples were centrifuged at 1200×g for 5 min at 4 °C, and then loaded on SDS–polyacrylamide gel (10.5–14% Tris–HCl, 4% stacking, Bio-Rad). The gel was run at 30 mA for 3 h using Tris–glycine running buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS). The separated proteins were transferred from the gel to a PVDF membrane (Roche) using a Mini-Blot electrophoretic transfer cell (Bio-Rad) and left to run overnight at 17 V. The membrane was then blocked with 2.5% dry milk in PBST for Western blotting (PBS buffer plus 0.1% Tween 20) for 2 h at room temperature. After a brief wash in PBST, the membrane was incubated with mouse monclonal antibody against VP7 (MAB8350, Chemicon), at a dilution of 1:1000 in 1% dry milk in PBST for 1 h at 37 °C. After a 15-min wash followed by three washes for 5 min, the membrane was incubated with horseradish peroxidase conjugated rabbit polyclonal IgG antibody against mouse (Bioscience, Beijing), diluted at 1:5000 in 1% dry milk in PBST. The membrane was then washed in PBST for 15 min followed by three washes for 5 min. A protein assay was performed using ECL Plus (Biosciences) according to the manufacturer’s instructions.

Enzyme-linked immunosorbant assay analysis (ELISA)

Total soluble protein was extracted from freeze-dried seedlings as previously described. The extracts were incubated in the ELISA plate (Bioscience, Beijing) for 2 h at 37 °C, followed by three washes with PBST by an ELISA (PBS plus 0.05% Tween 20) and blocking with 5% (w/v) dry milk in PBST for 1 h at 37 °C. After another three washes, mouse anti-VP7 monclonal antibody (MAB8350, Chemicon) (1:1000) was added and incubated for 2 h at 37 °C. After another three washes with PBST, the plate was incubated with peroxidase labeled rabbit anti-mouse antibody (1:2000 in PBST, Bioscience, Beijing), in which the final detection was performed using TMB peroxidase substrate (Bio-Rad). The reaction stopped after 5 min with 1 N H₂SO₄, in which the optical density was measured at 450 nm using the Microplate Reader Thermo Max (Bio-Rad).

Animals

Female BALB/c mice and female C57BL/6 mice (4–6 weeks old) were purchased from the Beijing Laboratory Animal Center (Beijing, China) and raised under clean conditions at the Laboratory Animal Resources Center of Third Military Medical University. Five mice were housed in an isolator cage, to which air is supplied directly and then exhausted. The mice were raised for 1 week before they were used for an experiment. Then, each mouse was placed in an individual cage before feeding the tubers. The mice had free access to sterile water and standard mouse chow except for the transgenic potatoes. Mice were monitored on a daily basis for signs of weight loss and other conditions. There were five mice subjected to each treatment.

Vaccination

The protocols are summarized in Table 1. Before an oral administration of potatoes, the mouse was fasted for 8 h. Then, the mouse was fed a diet containing 2 g of potato tubers (with 80 μg of plant-derived VP7) for 16 h.

Detection of VP7-specific immune responses

VP7-specific serum IgG and fecal IgA titers were measured and a plaque reduction neutralization assay was performed. In brief, 96-well ELISA plates (Corning, NY) were coated with 200 μl of 5 μg/ml rotavirus particles purified by sucrose gradient density centrifugation (about 300 ng VP7 protein per well) in PBS and incubated overnight at 4 °C. After three washes with PBST for an ELISA (PBS, pH 7.4, plus 0.05% Tween 20), plates were blocked with fetal bovine serum (FBS) in PBS (1:10) and incubated for 1 h at 37 °C. After three washes with PBST, serum was serially diluted twofold in the plates using FBS and starting at 1:100. The plates were subsequently incubated for 1 h at 37 °C before being washed three times with PBST and incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse-IgG1 (Southern Biotech), at a dilution of 1:4000 in blocking buffer (PBS, pH 7.4, plus 0.05% Tween 20), plates were blocked with HRP conjugated rabbit anti-mouse-IgG antibody (Bioscience), at a dilution of 1:2000 in blocking buffer, for 1 h at 37 °C. After washing with PBST fourfold, the detection was performed using TMB peroxidase substrate (Bio-Rad) for 5 min. The reaction was stopped with 1 N H₂SO₄, and then the absorbance was measured at 450 nm using the Microplate Reader Thermo Max (Bio-Rad). Titers were estimated as the reciprocal of the maximum dilution of serum giving in turn an absorbance reading of 0.1 U after the subtraction of non-specific binding in serum from non-treated animals (negative control).

Fecal samples were prepared by adding 5 ml of extraction buffer (PBS, 0.1% Tween 20 and 10 μg/ml leupeptin) into a gram of feces. Suspensions were kept for 30 min at 4 °C and thoroughly homogenized with a QBiogene Fast Prep machine for 1 min. After incubation for another 30 min at 4 °C, suspensions were centrifuged for 20 min at 16,000×g (Eppendorf), at 4 °C. The supernatant was collected and centrifuged again at 16,000×g at 4 °C for 1 min. The supernatant was collected and immediately analyzed by an ELISA or stored

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mouse type</th>
<th>Sample size</th>
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<tbody>
<tr>
<td>1</td>
<td>2 g of mock-transformed potatoes</td>
<td>Balb/c</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>2 g of VP7-transformed potatoes</td>
<td>Balb/c</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>2 g of VP7-transformed potatoes + adjuvant</td>
<td>Balb/c</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>2 g of mock-transformed potatoes</td>
<td>C57BL/6</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>2 g of mock-transformed potatoes</td>
<td>C57BL/6</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>2 g of VP7-transformed potatoes + adjuvant</td>
<td>C57BL/6</td>
<td>5</td>
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*10 micrograms of bacterial CTB, i.e., subunit B of cholera toxin.
at −20 °C for later use. Total soluble protein (TSP) of each sample was tested using the Bradford Assay (Bio-Rad). Twofold serially diluted samples of TSP, starting at 50 μg, were applied to the plates (previously coated with rotavirus and blocked as described in the section of serum samples) and incubated for 1 h at 37 °C. After washing in triplicate with PBST, plates were incubated for 1 h at 37 °C with an anti-mouse-IgA antibody (Sigma-Aldrich) diluted 1:1000 in a blocking buffer. Detection was performed as previously described for serum samples.

Neutralization assay

For the determination of neutralizing anti-HRV antibodies in sera and stools, sera and stools obtained from mice were tested by a microneutralization assay as described previously (Wu et al., 2003). Briefly, sera and stools from mice were heat-inactivated at 56 °C for 30 min and incubated with Wa strain rotavirus. A reduction in the number of HRV-infected cells of greater than 60% with respect to the number in control wells was indicative of neutralization. Neutralizing titers were greater than 60% with respect to the number in control wells yielding neutralization, as described.

Cytokine detection

In order to measure cytokine secretion, single splenocyte suspensions were prepared two weeks after the final booster. After 48 or 96 h of culture, cell-free supernatants were harvested and assayed. Supernatants were then screened for the presence of IFN-gamma, IL-2, IL-4, IL-5 and TGF-beta using sandwich-ELISA detection systems according to the instructions of the manufacturer (BMS) as described above.

Cytotoxicity assay

A standard 51Cr release assay (Heath et al., 1997) was used to measure CTL activity. As target cells, MC57 or P815 cells were infected with 100±130 m.o.i. of rotavirus Wa. After 5 h of incubation at 37 °C, the cells were trypsinized, washed, counted, centrifuged and labeled for 1 h at 37 °C with 50±100 mCi of Na51CrO4 (Zhongshan Company, Beijing, China). The cells were then washed in triplicate and placed in 96-well plates (10,000 cells/well). Effector cells were added at the appropriate concentrations in order to obtain various effector: target cell ratios (E:T) (12.5:1, 25:1 and 50:1) in a total volume of 200 μl. Spontaneous and total releases of 51Cr were calculated by incubating target cells in each experiment with medium only, or with 10% Triton X-100. After 5 h of incubation at 37 °C, the plates were centrifuged at 500×g for 5 min and 100 ml of supernatant was removed from each well and the gamma radioactivity was measured using a gamma counter (Spectra-Gamma, Packard Instruments Co., Meriden, CT). The percentage of specific lysis was calculated as 100×[(cpm released by CTL − cpm of spontaneous release)/(cpm of total release − cpm of spontaneous release)]. A response was considered positive when it was at least twice the lysis value of the control target cells (mock-infected and/or rotavirus-infected cells). The results were obtained from two or three experiments and each experiment was performed in triplicate. Results are expressed as mean±standard error of the mean (SEM).

Statistical analysis

All statistical analyses were performed using the software SPSS 11.0 for Windows. A two-tailed Student’s t test was used to compare the differences in mean values between groups. A P value less than 0.05 was deemed statistically significant.

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