

Interleukin-2 potentiates foot-and-mouth disease vaccinal immune responses in mice

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Received 26 May 2004; accepted 6 December 2004

Available online 24 December 2004

Abstract

The present study describes the role of recombinant human interleukin-2 (rh IL-2) as immunomodulatory molecule in foot-and-mouth disease (FMD) vaccinal immune response in a murine model. The humoral immune response was evaluated by examining the antibody titre against FMD virus type O, A₂₂ and Asia 1 in serum samples obtained from different groups of mice inoculated with PBS, FMD vaccine alone; vaccine along with rh IL-2 on 0, 7, 14, 21, and 30 days post vaccination (DPV) by indirect double antibody Sandwich ELISA. The cellular immune response was also examined on different DPV by an MTT based lymphoproliferation assay in splenic mononuclear cells (SMNC) obtained from different groups. IL-2 was able to enhance the specific immune response against FMD virus type O, A₂₂ and Asia 1 as evident by significantly higher ELISA antibody titres ($P < 0.05$) in serum obtained from mice receiving IL-2 along with vaccine as compared to mice immunized with vaccine alone. Similarly, the same group of mice showed significantly higher lymphoproliferative responses in SMNC against mitogen PHA and FMD virus types O, A₂₂ and Asia 1 on all DPVs as compared to the group inoculated with vaccine alone.

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Keywords: Foot-and-mouth disease; Oil adjuvant vaccine; IL-2; Mice; Immune response

1. Introduction

Foot-and-mouth disease (FMD) is an extremely contagious, acute viral disease of all cloven footed animals, characterized by fever and vesicular eruptions in mouth and on feet and teats. It is one of the world's most important animal disease because of the speed with which it spreads, trade sanctions imposed on countries in which it occurs and the loss in production of affected animals. The practice of regular prophylactic vaccination still continues as a primary control measure in many countries.

Humoral immune response was considered to be an important defence mechanism in FMD infection. In recent past, there has been an increasing evidence of the importance of

T cell mediated immunity in FMD [1]. The immunity provided by infection provides protection for sufficient period which appears to correlate with the presence of circulating specific antibodies [2]. While immunization with inactivated virus vaccine is reported to result in an immunity of shorter duration [3] which could be due to vaccine virus being less competent to induce a T cell response and T cell memory. Thus, effective adjuvant systems are very important for enhancement of immune response against FMD vaccines.

Although hundreds of substances have been found to have adjuvant effects, only few like aluminium hydroxide, saponin and oil emulsion have been routinely used in FMD vaccine [4]. In recent years, special attention has been paid to cytokines as vaccine adjuvants because of their unique roles in the immune response. Various cytokines have been shown to be effective as adjuvants in a variety of model systems, enhancing protection induced by viral, bacterial and parasitic

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vaccines [5]. Numerous *in vitro* and *in vivo* studies have revealed the importance of the local cytokine environment on the quality and magnitude of ongoing immune responses. The use of cytokines as adjuvants offers an important advantage as they will not only be able to enhance immune response in most appropriate manner but will also determine the type of immune response mounted by the antigen, i.e. an expanded B cell response, a cytotoxic T cell response or a T helper type 1 or 2 subset response depending on the cytokine that is being used. As IL-2 occupies a central position in the cascade of events involved in the immune response, it has been studied most extensively as potentiator of vaccination [6–13]. IL-2 has the capacity to induce the proliferation and differentiation of T cells as well as B cells and also stimulates the cytolytic activity of subsets of T lymphocytes, enhancing T cell activation and inducing secretion of other cytokines such as IFN- γ , IL-4 and TNF. It not only stimulates the proliferation of activated B lymphocytes but also promotes the induction of immunoglobulin secretion [14].

Limited work has been reported on inclusion of these immunomodulatory molecules in FMD vaccines for significant enhancement of the immune response and protection against FMD virus challenge. Based on these attributes, in the present study, the effects of IL-2 on immunomodulation of humoral as well as cellular immune responses against FMD vaccines was investigated.

2. Materials and methods

2.1. Mice

A total of 45 male outbred Swiss albino mice (3–4 weeks old) obtained from Disease Free Small Animal House, CCS Haryana Agricultural University, Hisar, India and maintained in pathogen free conditions were used.

2.2. Vaccine

Oil adjuvant polyvalent FMD vaccine (Raksha Ovac, Indian Immunologicals) containing binaryethyleneimine inactivated O, A₂₂, C and Asia 1 serotypes of FMD virus was used for immunizing mice.

2.3. Cytokine

Recombinant human interleukin-2 (rh IL-2, Sigma Chemicals) was used along with the vaccine.

2.4. Viral antigens

FMD virus (FMDV) reference serotypes O, A₂₂, C and Asia 1 (procured from Central Laboratory of Project Directorate on FMD; IVRI, Mukteshwar-Kumaon, Uttaranchal, India) were cultivated in baby hamster kidney (BHK-21) clone 13-cell line using minimum essential medium (MEM, GIBCO BRL) supplemented with lactalbumin hydrolysate

(LAH), tryptose soya broth, sodium bicarbonate, antibiotics and antifungal agents. The growth and maintenance media were supplemented with 10 and 2% fetal calf serum (FCS), respectively. The cells were infected at a multiplicity of infection (MOI) of 0.01 per cell and were harvested when they were showing virus specific CPE. The virus was extracted from the cells by freezing and thawing. Cell debris was removed by centrifugation at $10,000 \times g$ for 30 min at 4 °C. This preparation was used as antigen in ELISA. For lymphocyte proliferation assay antigens were inactivated with binaryethyleneimine [15].

2.5. Immunization

Three groups of 15 mice were each immunized (*i.m.*) on day 0 either with 0.1 ml of PBS (as negative control), or with 0.1 ml of FMD vaccine or with 0.1 ml FMD vaccine emulsified with 800 IU of IL-2, respectively. Blood and splenic mononuclear cells were collected from three mice of each group on 0, 7, 14, 21 and 30 days post vaccination (DPV).

2.6. Peripheral blood

Peripheral blood was collected from mice by use of the retro-orbital bleeding method. Serum was collected from these blood samples by placing the tubes in slanting position for 1 h at room temperature followed by 3–4 h incubation at 4 °C. The tubes were then centrifuged and serum was obtained by collecting the supernatant.

2.7. Indirect double antibody Sandwich ELISA

An ELISA to detect antibodies to FMD virus serotypes was carried out as indicated earlier [16]. The Micro ELISA polyvinyl plates (Nunc) were coated with 50 μ l of anti 146S rabbit sera (specific for the FMD virus serotypes under investigation) overnight at 4 °C. Plates were washed thrice with phosphate buffer saline containing 0.05% (v/v) Tween-20 (PBST), and 50 μ l virus antigen at a predetermined dilution was added and plates were incubated at 37 °C for 1 h. Plates were then washed three times with PBST. Thereafter, 50 μ l of double fold dilution of each test serum sample (in duplicate) in diluent buffer (LAH 3% (w/v), newborn calf serum 5% (v/v), normal rabbit serum 5% (v/v) in PBST) was added and incubated at 37 °C for 1 h. Following three washings with PBST, 50 μ l of optimally diluted anti-mouse IgG horse raddish peroxidase (HRPO) conjugate (Sigma) was added in all the wells and incubated further for 1 h at 37 °C. Finally, 50 μ l of freshly prepared substrate solution (orthophenylene diamine + hydrogen peroxide) was added to each well and plates were left in the dark for development of colour. The reaction was stopped by adding 50 μ l of 1 M sulphuric acid/well. The optical density of the wells were measured by an ELISA reader (Titertek Multiskan) using a measuring filter of 492 nm.

2.8. Estimation of titre

The titre was expressed as \log_{10} of the inverse of the highest serum dilution that had an O.D. greater than that obtained with negative serum ± 3 S.D.

2.9. Splenic mononuclear cells (SMNC)

Mouse spleens were passed through steel mesh to obtain single cell suspensions which were then slowly layered on top of Histopaque (1.077 ± 0.001 g/ml, Sigma) column and centrifuged at 1500 rpm for 30 min. After centrifugation, the white band of mononuclear cells was harvested and washed twice with RPMI-1640 medium. The cell pellets were resuspended in RPMI-1640 medium with L-glutamine supplemented with 10% FCS, 1% penicillin and 1% streptomycin. The viability of cells was estimated by the trypan blue dye exclusion technique and cell suspensions were adjusted to a final concentration of 1×10^6 cells/ml.

2.10. Culture of splenic mononuclear cells

SMNC suspension ($100 \mu\text{l}$; 1×10^6 cells/ml) was dispensed in triplicates into 96 well flat bottom microtitre plates. These cultures were then stimulated by adding $50 \mu\text{l}$ optimally diluted PHA (Gibco) or inactivated FMD virus types O, A₂₂ or Asia 1 antigens. The control wells contained $50 \mu\text{l}$ mock antigen in place of FMD virus antigens. Cultures were then incubated in a humid CO₂ chamber for 72 h at 37 °C. After incubation, $50 \mu\text{l}$ of 3[4,5-dimethylthiazol-2- μl]-2,5-diphenyltetrazolium bromide; thiazolyl-blue dye (5 mg/ml, MTT, Sigma) was added in each well and plates were further incubated at 37 °C for 4 h. Following incubation, the supernatant from each well was aspirated carefully and formazan crystals were solubilized by adding $100 \mu\text{l}$ DMSO containing 0.01% (v/v) Triton X-100 into each well. The absorbance of each well was then determined at a wavelength of 540 nm, calibration setting of 1.00 and a threshold of 1.99 and a reference wavelength of 620 nm. The ELISA reader was blanked with culture well having MTT alone without any SMNC. Δ O.D. was calculated by subtracting the mean O.D. of mock antigen stimulated cultures from the mean O.D. of specific virus stimulated cultures.

2.11. Statistical analysis

The results obtained on various parameters from the three experimental groups of mice were analyzed statistically using Duncan's multiple range test.

3. Results

3.1. Virus specific antibody responses

To see whether IL-2 when administered along with vaccine, is able to enhance the antibody titres against FMD virus antigens, the serum antibody titres against FMD virus types O, A₂₂ and Asia 1 were determined on 0, 7, 14, 21 and 30 DPV, using the Sandwich ELISA (Table 1). In the group of mice receiving IL-2 along with vaccine, the mean antibody titres were always significantly higher ($P < 0.05$) than the group, which was inoculated with vaccine alone. The mean antibody titre against FMD virus type O (Table 1) in the group receiving vaccine alone peaked on 14 DPV and thereafter remained static on 21 and 30 DPV where as in the group receiving IL-2 along with the vaccine, the antibody titres started increasing gradually after day 7 and reached peak on 21 DPV followed by a slight decline on 30 DPV while the peak antibody titres against FMD virus type A₂₂ (Table 1) and Asia 1 (Table 1) were obtained on 21 DPV. The antibody titres in the control group were always less than $1.0 \log_{10}$.

3.2. Lymphoproliferative responses in splenic mononuclear cells

The lymphoproliferative responses of SMNC obtained from the three experimental groups of mice in response to in vitro stimulation with mitogen PHA and different viral antigens were studied on 0, 7, 14, 21 and 30 DPV using MTT assay. The lymphoproliferative responses of PHA stimulated SMNC cultures in the group receiving IL-2 along with vaccine were significantly higher ($P < 0.05$) compare to the group receiving vaccine alone (Fig. 1a). The peak response in this group was observed on 14 DPV followed by a gradual decline on 21 and 30 DPVs. The lymphoproliferation in FMD virus types O (Fig. 1b), A₂₂ (Fig. 1c) and Asia 1 (Fig. 1d) stimulated SMNC cultures of mice immunized with IL-2 along

Table 1
Serum antibody titres (\log_{10}) in mice vaccinated with oil adjuvant polyvalent FMD vaccine or FMD vaccine + IL-2 against FMD virus types O, A₂₂, and Asia 1

Days post-vaccination	Control			FMD vaccine			FMD vaccine + IL-2		
	O	A ₂₂	Asia 1	O	A ₂₂	Asia 1	O	A ₂₂	Asia 1
0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
7	<1.0	<1.0	<1.0	1.0 ± 0.00 B	1.3 ± 0.00 B	1.4 ± 0.10 B	1.7 ± 0.10 A	1.8 ± 0.10 A	1.7 ± 0.10 A
14	<1.0	<1.0	<1.0	1.6 ± 0.00 B	1.4 ± 0.10 B	1.7 ± 0.20 B	1.9 ± 0.00 A	2.1 ± 0.10 A	2.2 ± 0.00 A
21	<1.0	<1.0	<1.0	1.6 ± 0.00 B	1.6 ± 0.00 C	1.9 ± 0.00 B	2.5 ± 0.17 A	2.3 ± 0.10 A	2.5 ± 0.00 A
30	<1.0	<1.0	<1.0	1.6 ± 0.00 B	1.6 ± 0.00 B	1.6 ± 0.00 B	2.2 ± 0.17 A	2.3 ± 0.17 A	2.2 ± 0.17 A

Values are mean \pm S.E. Mean with the same letters are not significantly different ($P < 0.05$).

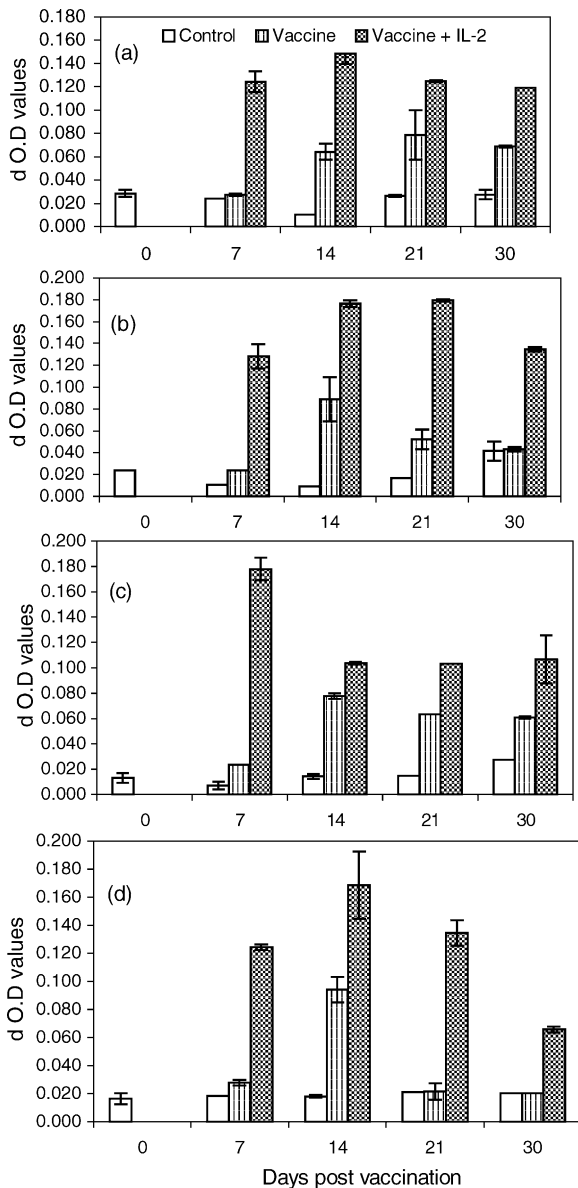


Fig. 1. Lymphoproliferative response of in vitro (a) PHA stimulated SMNC cultures, (b) FMD virus type O-stimulated SMNC cultures, (c) FMD virus type A₂₂-stimulated SMNC cultures and (d) FMD virus type Asia 1-stimulated SMNC cultures obtained from mice vaccinated with FMD vaccine or FMD vaccine along with rh IL-2.

with vaccine were significantly higher compared to those obtained in SMNC cultures of mice immunized with vaccine alone ($P < 0.05$). In cultures stimulated with FMD virus type O the peak lymphoproliferative response was observed on 21 DPV while in cultures stimulated with FMD virus type A₂₂, the peak Δ O.D. value was observed on 7 DPV followed by a decline on 14 DPV. However, the responses were then maintained at almost same level on 21 and 30 DPV. The trend in lymphocyte proliferative responses in the SMNC cultures on in vitro stimulation with FMD virus type Asia 1 was almost similar to that observed on stimulation with FMD virus type

O on different DPV except that the responses were of lower magnitude on 21 and 30 DPV.

4. Discussion

In the present study, significantly higher magnitude of antibody titres were recorded in the groups of mice which were immunized with FMD vaccine along with rh IL-2 as compared to the mice which received vaccine alone, i.e. up to 2.5 (\log_{10}) against FMD virus type A₂₂ antigen while in the group receiving vaccine alone highest titres against FMD virus type O and A₂₂ did not exceed 1.6 (\log_{10}) and against type Asia 1, highest titre recorded was 1.9 (\log_{10}). These findings clearly demonstrated that a single dose of IL-2 was able to augment the specific antibody responses when used as adjuvant with the polyvalent FMD vaccine. These results are consistent with the findings reported earlier that single concomitant injection of IL-2 (1000 IU/mouse) with virus antigen resulted in a significant anti-FMD virus antibody response at 14 and 28 DPVs [16]. Enhanced humoral immune response has also been demonstrated by several workers when multiple injections of IL-2 were used with inactivated rabies virus [17], BHV-1 subunit vaccine [9] and modified live BHV-1 vaccine [18]. Enhanced antibody responses to tetanus antigen [19] and influenza virus vaccine [20] by IL-2 liposomes has also been demonstrated.

IL-2 directly stimulates the proliferation of antigen activated B lymphocytes and also promotes the induction of immunoglobulin secretion [21]. The enhanced antibody titres may be because of the capacity of IL-2 to induce proliferation and differentiation of B cells as well as T cells [22]. IL-2 probably stimulated dramatic proliferation of the antigen specific T lymphocytes, which then further differentiated into mature antigen specific effector T helper cells. The proliferating T cells may then secrete a variety of other lymphokines like IL-4, IL-5 and IL-6 which then act in conjunction with T cell help to activate B cells and to generate mature antibody producing plasma cells and memory cells [8]. Thus, explaining the increased levels of specific antibodies in the mice which received rh IL-2 along with vaccine.

Generally, immune response against FMD was evaluated on the basis of humoral immune response, but recently, it has been suggested that cellular immune responses are also required for providing protection in animals [23]. In the present study, the adjuvant effect of rh IL-2 in enhancing the cellular immune response against FMD vaccine was also studied and our findings revealed that rh IL-2 could significantly augment the cellular immune response against FMD virus types O, A₂₂ and Asia 1 antigens. The lymphoproliferative responses in splenic lymphocyte cultures obtained from the group of mice inoculated with rh IL-2 were significantly higher on all days post vaccination as compared to the responses in group of mice receiving vaccine alone. Multiple doses of IL-2 have also been shown to enhance the antigen specific lymphoproliferative responses when given in conjunction with BHV-1

subunit vaccine based on glycoprotein IV [9]. Several other workers [7–9] have reported that IL-2 increases the cellular immune responses but they attributed it to augmented antigen specific cytotoxicity reactions.

The significantly higher proliferative responses in the group of mice inoculated with rh IL-2 along with vaccine may be because of larger number of responding antigen specific T cells in the splenic mononuclear cells. Once even a small number of these T cells are activated, they might be secreting GM-CSF along with other immunostimulatory factors like IFN- γ and IL-4, which might then augment the number as well as efficiency of active antigen presenting cells, which in turn would be able to recruit additional antigen specific T cells. This positive feed back loop may thus be resulting in an efficient antigen specific cellular immune response.

It can be well concluded from the present study that rh IL-2 can significantly augment the humoral as well as cellular immune response against FMD. A further enhancement of the specific immune response against FMD virus may be expected if the recombinant cytokines of the same species are used as adjuvants.

However, further studies are needed in natural hosts to optimize the dose of antigen as well as that of cytokines and the route and schedule of inoculation. It has been reported that IL-2 when administered in conjunction with vaccine elicited a response similar to that generated by immunizing with 25 times as much antigen [8–24]. Thus, if a formulation capable of releasing IL-2 slowly becomes available, then the use of this cytokine as an adjuvant would become a more practical proposition in the field.

It has been observed that IL-2 when administered in oil adjuvant can overcome the MHC-linked genetic non-responsiveness to antigens [25], which is also a major problem encountered while controlling FMD by vaccination. Thus, further studies may be carried out to investigate this beneficial effect of IL-2 with FMD viral antigens.

The results of present study would be of major importance if recombinant cytokines such as IL-2 when used as adjuvants along with the synthetic or recombinant structural proteins of FMD virus enhance the immunogenicity of these antigens.

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