REPLICATION OF JAPANESE ENCEPHALITIS VIRUS IN MOUSE BRAIN INDUCES ALTERATIONS IN LYMPHOCYTE RESPONSE

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Summary. – The experimental model using intracerebral (i.c.) challenge was employed in many studies evaluating the protection against disease induced by Japanese encephalitis virus (JEV). We investigated alterations in peripheral lymphocyte response caused by i.c. infection of mice with JEV. Splenocytes from the i.c.-infected mice showed suppressed proliferative response to concanavalin A (con A) and anti-CD3 antibody stimulation. At the same time, the expression of CD25 (IL-2R) and production of IL-2 was inhibited. Addition of anti-CD28 antibody restored the decreased anti-CD3 antibody-mediated proliferation in the splenocytes. Moreover, the number of con A-stimulated cells secreting IL-4 was significantly reduced in splenocytes from i.c.-infected mice. These studies suggested that the i.c. infection with JEV might involve additional immune modulation effects due to massive virus replication in the brain.

Key words: Japanese encephalitis virus; intracerebral infection; immune response

Introduction

JEV is a positive-sense single-stranded RNA virus that belongs to the family *Flaviviridae,* genus *Flavivirus* (Heinz and Allison, 2000; Lindenbach and Rice, 2003) and is associated with encephalitis and damage to the central nervous system due to its neurotropic nature (Westaway *et al*., 1995; Oyanagi *et al*., 1969; Kimura-Kuroda *et al*., 1993). Although many aspects of flavivirus infection relating to host immune responses have been studied, the consequence of JEV infection in the brain that is a relatively immunologically privileged site on the T lymphocyte responsiveness is not completely understood. Surprisingly, only a few studies investigated the immune response to JEV during acute phase of brain infection. Conversely, many protection experiments testing the efficacy of vaccination are based on the i.c.-challenge model using viral doses exceeding several times the LD_{50} dose.

Previously we reported that i.c. but not peripheral injection of JEV led to induction of MHC-I on thymocytes (Kavitha and Manjunath, 2004). Therefore, we wished to examine the effects of i.c. JEV infection on peripheral lymphocytic responses. In current study, we report that i.c. infection of BALB/c mice with JEV causes a decrease in the mitogen-stimulated proliferation and IL-4 secretion of splenocytes from infected mice. In addition, the levels of anti-CD3 antibody-stimulated IL-2R, but not lymphocyte function-associated antigen 1 (LFA-1) decreased significantly in splenocytes from i.c.-infected mice suggesting that i.c. infection with JEV could lead to altered lymphocytic responses in the periphery.

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Abbreviations: APC(s) = antigen presenting cell(s); con A = concanavalin A; CTL = cytotoxic T lymphocyte; i.c. = intracerebral; i.p. = intraperitoneal; i.v. = intravenous; JEV = Japanese encephalitis virus; LFA-1 = lymphocyte function-associated antigen 1; MAbs = monoclonal antibodies; MFI = mean fluorescence intensity; OVA = ovalbumin; p.i. = post infection; PMA = phorbol-12-myristate-13-acetate; TCR = T lymphocyte receptor

Materials and Methods

Cells and viruses. JEV strain P20778, an isolate from human brain (Kedarnath *et al*., 1986) was routinely grown in a mosquito cell line C6/36 maintained in MEM (Sigma) supplemented with 10% FBS (Gibco) and infected at a MOI of 1. Infected C6/36 cell culture supernatants served as the source of virus in all studies after titration by infectious plaque assays on porcine kidney (PS) cells (Murali-Krishna *et al*., 1994). To inactivate virus, virus-containing supernatants were heated at 60°C for 1 hr.

Lymphocyte cells were maintained in RPMI 1640 medium (Sigma) supplemented with non-essential amino acids (1 mg/ml), sodium pyruvate (12 mg/ml), L-Glutamine (0.32 mg/ml), β-mercaptoethanol (5×10^{-5} mol/l), penicillin (100 U/ml), streptomycin (250 μg/ml), gentamycin (50 μg/ml), (all chemicals were purchased from Himedia laboratories, India) and 5% FBS (Gibco). RPMI 1640 containing 0.5% FBS was used for washing of cells. Spleen cells (1×10^5) were cultured in 0.2 ml complete RPMI medium in the presence of con A (Sigma, 0.2 μg/well), anti-CD3 antibody (8 μl/well), antibodies anti-CD3 and anti-CD28 combined (25 μl/well) or PMA (Sigma, 10 nmol/l) with ionomycin (Sigma, 500 nmol/l). [^{3}H]thymidine (Brit, India, 1 μCi/well) was added 10 hrs before harvesting of cells. The data were presented as mean Δcpm ± SD obtained after subtracting the cpm values from non-stimulated cultures.

For the preparation of JEV antigen, virus-infected P388D1 cells (H-2^d, DBA/2 derived mouse macrophage, syngeneic to BALB/c) at MOI 10 were cultured for 48 hrs, washed, and treated with 0.025% glutaraldehyde (Sigma) in PBS for 15 mins at 4°C. The cells were washed, sonicated (Sonics Vibra cell), and centrifuged at 500 × g for 10 mins. The supernatant served as JEV antigen for proliferation assays. Control antigen was prepared from non-infected P388D1 cells.

CTLL-2 cells used in bioassays to quantify IL-2 were routinely grown in RPMI 1640 medium that was supplemented with 5% FBS and exogenously added 20 U/ml recombinant IL-2.

YAC-1 cells (H-2^a, A/J mouse derived T cell leukemia) that are used routinely as target cells for mouse natural killer cells in ^{51}Cr release assays were grown in RPMI 1640 containing 5% FBS. The irradiation of cells used in lymphocytic proliferation and CTL generation studies was performed at 3,500 Rads using a ^{60}Co radiation source (BRIT, India).

Infection and immunization of mice. BALB/c (H-2^d) mice 6–8 weeks old were subjected to mild ether anesthesia and injected with 400 PFU of JEV by the i.c. route. Acute infection of mice with JEV, strain P20778 leading to death could be performed only by direct i.c. route and not by intraperitoneal (i.p.) or intravenous (i.v.) route. Immunization (i.p.) of mice was performed with PBS (control) or with JEV-infected porcine kidney cells (JEV-PS) (Murali-Krishna *et al*., 1994). Both groups of mice were i.c.-challenged with 400 PFU of JEV on day 10. Mice immunized with virus-infected cells were protected from death caused by i.c. challenge with JEV. Ovalbumin (OVA) as protein control (150 μg) emulsified in Freund's adjuvant was i.p.-injected, and 21 days later the mice were i.c.-challenged with JEV. All mice challenged with JEV were sacrificed by cervical dislocation on day 9 post infection (p.i.).

Antibodies. Mouse monoclonal antibodies (MAbs) anti-K^d/D^d (34-1-2s), anti-flavivirus group-specific (D1-4G2-4-15), and anti-Thy 1.2 (HO-13-4), rat MAbs anti-mouse CD25 (PC61-5.3), and hamster MAbs anti-mouse CD28 (37.51), anti-mouse CD3 (145-2C11) were obtained as hybridoma cell culture supernatants and used as primary antibody. Rat MAbs: FITC-conjugated anti mouse CD4 (GK 1.5), anti mouse CD8 (53-6.7), anti-LFA-1 (M17/4.2), PE-conjugated anti mouse MHC-II, IA/IE (M5/114.15.2), and the appropriate FITC- and PE-conjugated isotype control antibodies were obtained from BD Pharmingen, FITC-goat anti-mouse IgG (Fcγ fragment-specific) from Jackson Immunoresearch, and preadsorbed FITC-goat anti-rat IgG (Fcγ fragment-specific) from Sigma.

Isolation of non-T cells. Non-T cells were isolated from splenocytes of BALB/c mice by complement-mediated depletion of T cells and used as antigen presenting cells (APCs) in lymphocyte proliferation studies. The splenocytes from each spleen were resuspended in RPMI 1640 with 5% FBS, passed through a nylon mesh, and centrifuged. The pellet was resuspended in the mixture of hybridoma culture supernatants containing anti-thy1.2, anti-CD4, and anti-CD8 MAbs and incubated for 1 hr on ice. Then, the cells were centrifuged and the cell pellet was resuspended in 4 ml of media containing freshly diluted (1:40) rabbit complement. After incubation at 37°C for 30 mins, dead cells and debris were removed by centrifugation on Ficoll-hypaque-1083 (Sigma) at 700 × g for 15 mins. Viable cells seen at the interface were washed, resuspended in the complete medium and used as non-T cells. Adherent splenocytes were isolated by adherence to plastic plates at 37°C/5% CO_2 for 2 hrs.

FACS. Splenocyte suspensions in RPMI 1640 with 0.5% FCS medium were washed twice and 10^6 cells were analyzed for cell surface markers CD25 (IL-2R), LFA-1, MHC-I, and MHC-II (Kavitha and Manjunath, 2004). The control cells were stained only with secondary antibody or with isotype control antibodies when direct primary conjugates were used. First, titration studies were performed with all the antibodies used to ensure optimum fluorescence. The data were acquired by a FACScan Cytometer (Becton Dickinson, USA) for 10,000 events for each sample using WinList software (version 4.0) after appropriate gating to exclude dead cells and debris based on forward and side scatter profiles.

Lymphocyte stimulation and proliferation. Antigen specific proliferation of cells was performed with splenocytes (3×10^5) obtained from mice immunized with JEV-infected cells and stimulated with varying concentrations of JEV antigen for 72 hrs. Irradiated splenocytes from control mice (1×10^5) were included as an additional source of APCs since their addition resulted in optimal proliferation. After cultivation at 37°C for 48 hrs, the cells were harvested on glass fiber filters and counted in a LKB Pharmacia liquid scintillation counter. Data were represented as % of mean cpm ± SD of triplicates obtained for control con A-stimulated splenocytes after subtracting the value for non-stimulated cells.

Transwell and IL-2 assay. In transwell (Millicell TM HA, Millipore) experiments, 1×10^5 splenocytes from control or i.c.-infected mice were added in 0.2 ml complete medium to the upper chamber that was separated from the lower chamber by a 0.45 μm membrane filter. 1 μg/ml con A was added to the lower chamber in a total volume of 0.6 ml of medium. In order to determine the

necessity for cell contact to increase proliferation, irradiated non-T cells obtained from control splenocytes (6×10^5 cells) were added to the lower or upper chamber as APCs. The cells were incubated further and 0.1 ml aliquots from upper and lower chambers were transferred to 96 well plates, pulsed with ^{3}H-thymidine and incorporation of radioactivity was measured. Data were represented as % of mean cpm values of duplicates obtained for control con A stimulated splenocytes.

IL-2 production in culture supernatants was evaluated using an IL-2 specific cell line CTLL-2 maintained in IL-2 medium. The cells were washed twice with medium to remove residual IL-2 and added to 96-well plates at 5×10^3 cells per well along with supernatants obtained from splenocyte cultures or with appropriate serial dilutions of recombinant IL-2 (1.5 to 14 units). Control wells contained cells only. The standard proliferation curve obtained from the 36 hrs culture with added IL-2 was linear over the range of 1.5 units to 14 units yielding 5,000 to 10,0000 cpm. Data were represented as mean ± SD of IL-2 U/ml of culture supernatants.

CTL generation and ^{51}Cr-release assays. Cytotoxic T lymphocytes (CTL) against JEV were raised by i.p. injection of mice with JEV-infected cells or by the direct i.c. injection of 400 PFU of JEV into naïve mice. Both groups of mice were sacrificed 9 days p.i. and splenocytes were stimulated *in vitro* with JEV-infected P388D1 cells for 4 days (Murali-Krishna *et al*., 1994). Virus-specific CTL activity was detected using ^{51}Cr-labeled infected and non-infected P388D1 cells. Triplicates were used for each effector/target ratio and the % of ^{51}Cr-release was calculated after subtracting spontaneous leakage of ^{51}Cr. Spontaneous release of ^{51}Cr was 21% (P388D1 cells), 33% (JEV-P388D1cells) and 17% (YAC-1 cells). Values are expressed as mean % of ^{51}Cr-release ± SEM.

ELISPOT assay. Elispot kits for murine IFN-γ and IL-4 were obtained from Cell Sciences and Becton Dickinson. Splenocytes were obtained from i.c.-injected or control mice on day 3, 5, 7 or 9 p.i. Cells (5×10^5) were cultured in Elispot plates in the absence or in the presence of 5 μg/ml con A or 1 ng/ml PMA with 500 ng/ml ionomycin for 24 hrs and Elispots for IL-4 and IFN-γ were developed. The number of secreting cells in response to stimulation were determined within a linear range, standardized to 1×10^6 spleen cells and represented as mean ± SD.

Results

Decreased proliferation of splenocytes upon i.c. infection

Many viral infections are characterized by decreased T cell proliferative responses to different stimulating agents (Hutchings *et al*., 1990; Saron *et al*., 1991; Hsu *et al*., 2001). Thus, the proliferation of lymphocytes was evaluated in the i.c.-infected mice. Splenocytes were isolated from non-infected (control) and i.c- infected mice on day 9 p.i. and stimulated with an optimal dose of con A (0.2 μg/well), the mitogenic lectin. A significant inhibition was observed in the proliferation of splenocytes from i.c.-infected mice that was dose dependent and decreased with increasing doses of 0.4 and 0.8 μg/well of con A (Table 1).

Since IL-2 secretion is implicated in con A induced T cell proliferation (Kim *et al*., 2001) and activation, splenocytes from i.c.-infected mice were activated with con A and the production of IL-2 was measured in culture supernatants. Control cells from non-infected mice produced 200 U/ml of IL-2, while splenocytes from i.c.-infected mice produced barely detectable levels of IL-2 (Table 1). Amount of IL-2 was calculated from a standard proliferation curve obtained with different concentrations of IL-2. Based on these results, exogenous addition of IL-2 along with con A (0.2 μg/well) restored the deficiency in proliferation of splenocytes obtained from i.c.-infected mice. These results suggested

Table 1. Lymphocyte proliferation in control and JEV-infected mice

Stimulation[a]	Control	Infected	Inhibition[b] (% of control)
	[^{3}H]thymidine incorporation (Δcpm ± SD)		
Con A 0.2 μg	113166 ± 4341	13066 ± 1680	11.5***
0.4 μg	247158 ± 7831	112176 ± 8791	45.4***
0.8 μg	256198 ± 11654	207165 ± 12519	80.9*
Con A (0.2 μg) + rIL-2 (10 U/ml)	110174 ± 11746	117448 ± 10151	107.0
IL-2 production (U)			
Con A (0.2 μg)	202 ± 11.8	5.9 ± 1.2	2.9***
None	<4	<4	0

[a]Splenocytes from mice infected i.c. with 400 PFU of JEV and control mice were stimulated with given substances.
[b]The significance of inhibition is shown by *P* values of <0.05 (*), <0.005 (**), <0.0005 (***).

Table 2. Antigen-specific lymphocyte proliferation in control and JEV-infected mice

Stimulation[a]		[^{3}H]thymidine incorporation (Δcpm ± SD)		Inhibition[b] (% of control)
		Control	Infected	
Anti-CD3		162923 ± 4977	32580 ± 1063	19.9***
Anti-CD3 + Anti-CD28		259348 ± 21971	212821 ± 14005	82.1*
PMA + ionomycin		231504 ± 10906	236529 ± 24741	102.0
Unimmunized	4.50 µg	10448 ± 319	7956 ± 168	76.1**
		Immunized i.p.		
JEV antigen	0.60 µg	60221 ± 5870	31109 ± 4637	51.7*
	2.25 µg	87554 ± 1109	57303 ± 2374	65.4*
	4.50 µg	103771 ± 5187	66308 ± 1962	63.8*
Ovalbumin	5.0 µg	19862 ± 1658	3100 ± 205	15.6**
	12.5 µg	24769 ± 5054	2480 ± 168	10.0***
	25.0 µg	35381 ± 1812	2051 ± 375	5.8***

[a]Splenocytes from mice infected i.c. with 400 PFU of JEV and control mice were stimulated with given antigens.
[b]The significance of inhibition is shown by *P* values of <0.05 (*), <0.005 (**), <0.0005 (***).

that splenocytes from i.c.-challenged mice were unable to proliferate optimally in the presence of con A at day 9 p.i. possibly due to decreased availability of IL-2.

Furthermore, the decreased proliferation was observed when T cell receptor (TCR) was stimulated with an optimal concentration of anti-CD3 antibody. Splenocytes from i.c.-infected mice showed decreased proliferation when stimulated with anti-CD3 antibody (Table 2). This decrease was significantly corrected by the addition of anti-CD28 antibody suggesting that co-stimulation was sub-optimal in splenocytes from i.c.-injected mice. However, maximal stimulation of cells using PMA/ionomycin was the same in control as well as i.c.-infected splenocytes suggesting that the ability of these cells to respond to external stimulation was not compromised. We tested also mice i.v.-challenged with JEV, but they did not significantly alter con A and anti-CD3 stimulation of splenocytes (data not shown).

Antigen-specific proliferation of splenocytes obtained from immunized mice was also tested (Table 2). The viral antigen-stimulated proliferation of splenocytes from non-immunized control and i.c.-challenged mice mice was significantly low. Immunization of mice with virus-infected cells resulted in a significant increase in the dose-dependent, antigen-specific proliferation of splenocytes. Following i.c. challenge of immunized mice with JEV, subsequent *in vitro* priming of the splenocytes with antigen could elicit only a significantly low proliferative response although it was antigen dose-dependent. The increase in proliferation due to JEV-specific stimulation observed with 4.5 µg antigen was 9.9 and 8.3-fold in the immunized control and i.c.-challenged mice, respectively, compared to their non-immunized non-challenged counterparts (Table 2). The above data indicated that dose-dependent, JEV-specific proliferative responses were present in immunized, i.c.-challenged animals, although they were significantly lower when compared to non-challenged immunized mice. The effects of i.c. challenge were more apparent when an unrelated antigen such as OVA was used for i.p. immunization. Splenocytes from OVA-immunized, i.c.-challenged mice failed to show significant dose-dependent recall responses to OVA, when compared to OVA-immunized but non-challenged controls (Table 2). These results suggested that the ability of splenocytes from i.c.-challenged mice to proliferate was deficient in antigen recall assays.

Decreased production of IL-2, IL-2R but unchanged expression of LFA-1

Expression of cell surface markers IL-2R, LFA-1, MHC-I, and MHC-II were tested on splenocytes from control and i.c.-infected mice. Since IL-2 secretion from con A stimulated splenocytes decreased in i.c.-challenged animals and IL-2 availability regulates the expression of IL-2R (Kim *et al*., 2001), the presence of CD25 (IL-2R) was measured on anti-CD3 antibody stimulated splenocytes. The activation of splenocytes with anti-CD3 antibody for 48 hrs upregulated IL-2R to 73% of the level of control splenocytes (Fig. 1, IA). However, only 30% of cells obtained from i.c.-challenged mice stained specifically for CD25 upon anti-CD3 activation (Fig. 1, IIA). In contrast, LFA-1 expression did not decrease but showed a modest increase on anti-CD3 stimulated splenocytes obtained from i.c.-challenged mice (Fig. 1B). Flavivirus infections of cells *in vitro* upregulate expression of several adhesion molecules on the cell surface

(King *et al*., 2003). The constitutive expression of LFA-1 observed without anti-CD3 stimulation remained unchanged in non-infected and i.c.-infected mice while CD25 expression was the same as the isotype control (data not shown).

Cell surface levels of MHC-II regulate exogenous antigen presentation and decreased MHC-II expression could lead to the decreased secretion of IL-2. Therefore, cell surface MHC-II expression was analyzed on adherent non-stimulated splenocytes obtained from i.c.-infected mice (Fig. 1C). Adherent splenocytes were separated from total splenocytes in order to enrich them for cells expressing MHC-II. Although the total number of cells positive for MHC-II expression remained unchanged, the mean fluorescence intensity value (MFI) decreased on adherent splenocytes isolated from i.c.-infected mice (MFI = 1028 vs. MFI = 484). However, no significant changes in MHC-I expression occurred on splenocytes from control or i.c.-infected mice (Fig. 1D). These results indicated that the anti-CD3 stimulated expression of CD25, but not expression of LFA-1 was reduced in splenocytes from i.c.-infected mice. Additionally, MHC-II expression decreased on adherent cells, while MHC-I expression remained unaltered on total splenocytes.

Non-T cells from control mice restored the defect in proliferation of i.c.-infected splenocytes

The data presented above suggested that inadequate signaling and IL-2 secretion from T cells could be one of the reasons for decreased proliferation of splenocytes from i.c.-infected mice. Thus, the non-T cell population from control splenocytes was prepared and added to the splenocytes of i.c.-infected mice in order to examine changes in the deficiency of proliferation. Non-T cells from control mice were prepared using antibody-dependent complement depletion. The purity of obtained non-T cells was 90% as judged from surface Ig staining for B cells by FACScan (data not shown). This enriched population of non-T cells is likely to contain besides B cells also other APCs such as macrophages. The APCs from control mice were irradiated and added to the splenocytes from i.c.-infected mice that were stimulated with 1.0 μg/ml of con A (Fig. 2). Addition of 0.1, 0.5 and 1.0×10^5 of irradiated control APCs improved the proliferation of splenocytes from i.c.-injected mice in a dose-dependent manner. Cells were cultivated for 48 hrs and pulsed with ^{3}H-thymidine for 10 hrs before harvesting. On the other hand, irradiated APCs (1.0×10^5) prepared from the spleen of i.c.-injected mice were also tested on control splenocytes, but no change in proliferation of control splenocytes was observed suggesting that APCs from i.c.-injected mice did not have inhibitory effect on the proliferation of control splenocytes (Fig. 2). Hence, the proliferative capacity of i.c.-infected splenocytes could be

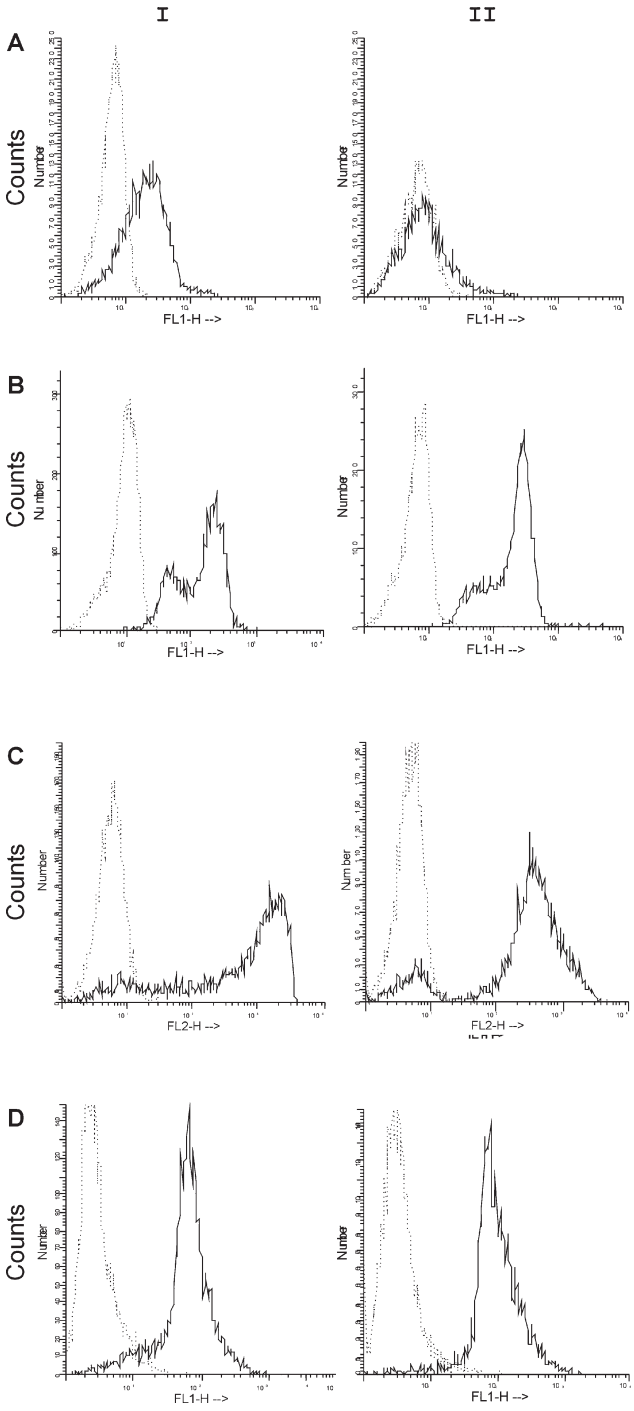

Fig. 1

Expression of cell surface markers IL-2R (A), LFA-1 (B), MHC-II (C) and MHC-I (D) on splenocytes obtained from control (I) and i.c.-infected mice (II) detected by FACS analysis

Solid and dashed lines indicate histograms obtained with specific and control antibody, respectively.

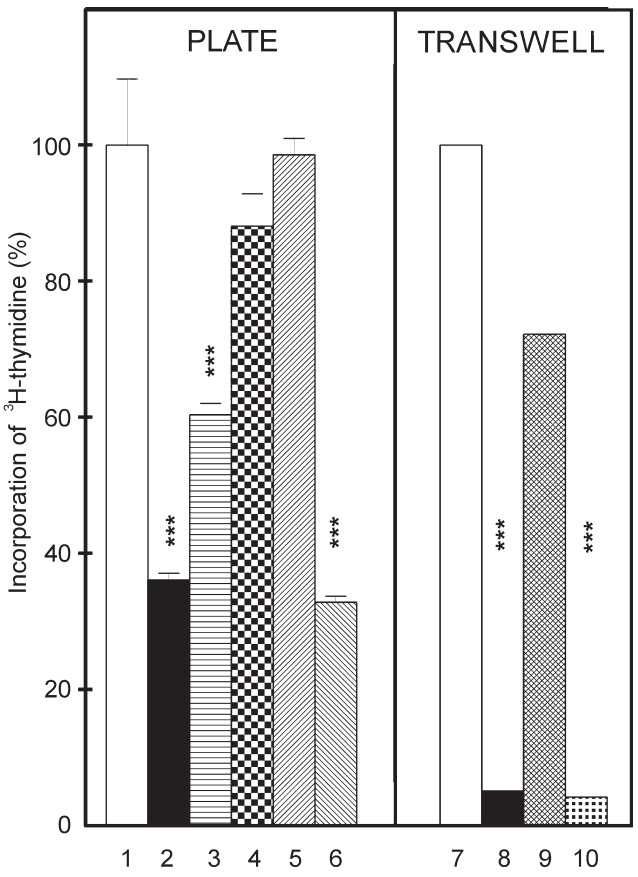

Fig. 2

Restoration of splenocyte proliferation obtained from i.c.-challenged mice

Left panel: i.c.-infected splenocytes cultivated with addition of 0.1×10^5 (3), 0.5×10^5 (4) and 1.0×10^5 (5) non-T cells. Control splenocytes with addition of 1.0×10^5 of i.c.-infected splenocytes (6). Control (1) and i.c.-infected (2) stimulated splenocytes. Right panel: transwell experiment – control splenocytes (7), i.c.-infected splenocytes (8), i.c.-infected splenocytes cultivated with APC in the same chamber (9) or in separate chambers (10). Values are represented as *** $P < 0.0005$.

restored when control APCs were exogenously added to the culture.

The restoration of proliferation by addition of control APCs was cell-contact dependent as shown in the transwell experiments. The restoration of proliferation of splenocytes from i.c.-infected mice was observed only in case that both the irradiated APCs and the responding cell population were added to the same transwell chamber. The restoration of proliferation was absent when the two types of cells were added separately to the transwell chambers divided by the membrane (Fig. 2).

Decrease in IL-4 producing cells

The impaired proliferation and antigen presentation could lead to an imbalance in cytokine secretion. The imbalance in helper T cell type 1 (T_H1) and type 2 (T_H2) cytokines is known to cause alterations in the pathogenesis of acute viral infections. Therefore, we studied the secretion of IFN-γ (T_H1 cytokine) and IL-4 (T_H2 cytokine) in splenocytes of i.c.-infected and control mice. The splenocytes were activated with con A or PMA/ionomycin and the number of cells producing IL-4 or IFN-γ was detected by Elispot assay (Fig. 3).

Splenocytes from control or i.c.-infected mice did not produce any IL-4 without the stimulation. A significant number of IL-4 producing cells was detected in control splenocytes upon stimulation with con A, but a very low number was observed in stimulated splenocytes of i.c.-infected mice on day 9 p.i. (Fig. 3A). However, no difference in the number of IL-4 producing cells was observed between control and i.c.-infected splenocytes when PMA/ionomycin was used to stimulate the cells suggesting that the splenocytes obtained from i.c.-injected mice were capable of responding to the external stimuli. The frequency of IFN-γ secreting cells was also low in the con A-stimulated splenocytes from i.c.-infected mice on day 9 in comparison with control mice. However, the extent of reduction was much lower than the change observed in the number of IL-4 producing cells from i.c.-infected mice (Fig. 3B). Again, no significant difference in the IFN-γ production was observed between control and i.c.-infected mice, when the splenocytes were stimulated with PMA/ionomycin. The cytokine secretion pattern of i.c.-infected splenocytes at earlier days 3, 5, 7, p.i. did not show any significant changes when compared to control splenocytes. These experiments showed that i.c. infection with JEV resulted in altered cytokine secretion in con A stimulated splenocytes.

CTL generation in splenocytes upon i.c. infection

Splenocytes obtained from mice that were either immunized with JEV-infected cells or directly i.c.-infected with JEV generated CTL upon co-cultivation with JEV-infected syngeneic P388D1 cells. At the ratio of the effector to target cells of 40:1, JEV-infected P388D1 cells were lysed by CTL from immunized mice to an extent of $63.3 \pm 1.2\%$, while the lysis of control non-infected P388D1 cells was approximately $6.3 \pm 0.7\%$. The virus-specific lysis (57%) obtained after subtracting of control cell lysis was similar to the virus-specific lysis (60.7%) obtained with CTL generated from splenocytes of i.c.-infected mice (Fig. 4). The effector cells causing target cell lysis were shown to be CD8 positive in our previous study (Murali-Krishna *et al.*, 1996). The natural killer activity obtained on YAC-1 target cells was negligible for both types of effectors.

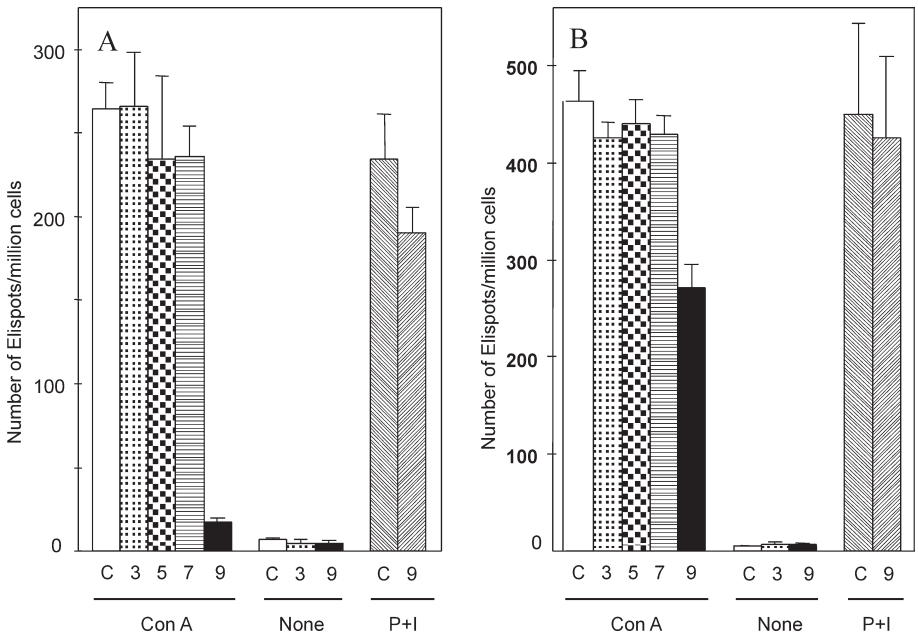

Fig. 3

IL-4 (A) and IFN-γ (B) production detected by Elispot assay

Control (C) or i.c.-infected splenocytes at day 3 (3), day 5 (5), day 7 (7), and day 9 (9) were cultivated in the absence (none) or presence of con A or PMA with ionomycin (P+I).

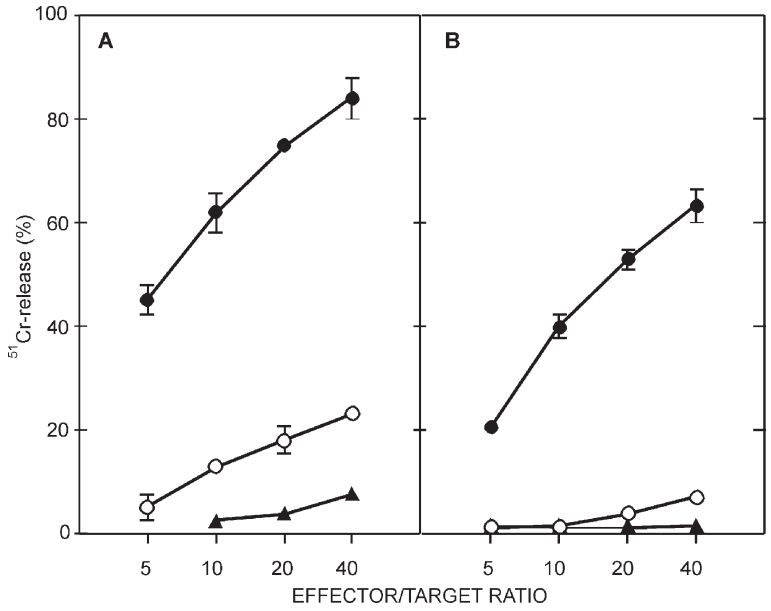

Fig. 4

Generation of JEV-specific CTL in mice immunized with JEV-infected cells (A) or i.c.-challenged with JEV (B)

The CTL generated were assayed with ^{51}Cr-labeled JEV-P388D1 (•), P388D1 (o), and YAC-1 (▲) target cells. Data represent one of three independent experiments.

Discussion

We report here that splenocytes obtained from mice 9 days after i.c. challenge with JEV showed decreased proliferation when stimulated with the mitogen, con A or anti-CD3 antibody. A number of mechanisms concerning impaired antigen presentation or IL-2 secretion have been proposed to explain decreased virus-induced lymphocyte proliferation (Mathew *et al*., 1999; Fugier-Vivier *et al*., 1997). To analyze further the effects of i.c.-challenge with JEV, we studied the alterations in lymphocytic responses occurring in the spleen cell population that could contribute to the observed decrease in proliferation. The IL-2 production from con A-activated splenocytes was reduced in i.c.-infected mice and inadequate IL-2 production could contribute to decreased proliferation of cells. The restoration of the proliferative defect in i.c.-infected splenocyte cultures upon addition of exogenous IL-2 supports this possibility. Since the activation of T cells results in secretion of IL-2 (Lin and Leonard, 1997), the decrease in IL-2R expression levels as well as the absence of recall responses to OVA in i.c.-infected mice could also be due to the observed defect in IL-2 production. Recall responses to JEV antigen in mice that were immunized with virus-infected cells and subsequently i.c.-challenged were also lower in magnitude than in their unchallenged counterparts. The presence of recall responses in the case of JEV antigenic stimulation but not OVA stimulation could be due to virus-specific stimulation caused by intracerebral replication of JEV and release of viral antigen into circulation.

Co-stimulatory signals generated through CD28-B7 interactions play a critical role in propagating the primary T cell receptor mediated signal during T cell activation (Lenschow *et al*., 1996; Schwartz, 1999). Stimulation of cells with anti-CD28 antibody leads to an increase in IL-2 expression (Harding *et al*., 1992; Thompson *et al*., 1989) and accordingly, addition of anti-CD28 antibodies increased the proliferation of splenocytes from i.c.-infected mice that were stimulated with anti-CD3 antibody. Our conclusion that the CD28-B7 interaction was suboptimal in i.c.-infected mice is supported by the restoration of the proliferative capacity in i.c.-infected splenocytes with exogenous addition of control APCs, the necessity for cell contact for this restoration and decreased B7 expression that we have observed in other experiments with peritoneal exudate cells obtained from i.c.-challenged mice. Higher concentrations of con A were observed to induce optimal proliferation in splenocytes obtained from i.c.-injected mice. This gives additional support to the conclusion that T cells from i.c.-infected mice could be defective in receiving the second signal, since stimulation of naïve T cells requires a co-stimulatory signal (Perrin *et al*., 1997) at lower but not at higher concentrations of con A. As well as, the addition of higher concentrations of JEV antigen tended to increase the antigen-specific stimulation in proliferation of i.c.-challenged splenocytes suggesting that the proliferative defect could be corrected at higher antigenic doses.

The mice injected i.c. with 400 PFU of virus succumbed to the infection in 9–12 days. Although maximal effect was observed with splenocytes obtained at this time, they could be the result of earlier events caused by the rapid multiplication of the virus within the brain and not entirely due to the morbidity. In fact, our experiments showed that exogenous addition of control APCs could restore proliferation of i.c.-infected splenocytes obtained from mice on day 9 p.i. Thus, they could respond normally when provided with the appropriate stimuli. The restoration of proliferation and cytokine secretion in i.c.-injected cultures with exogenous addition of PMA/ionomycin also supports the argument that the splenocytes obtained from mice on day 9 p.i. were capable of responding to external stimuli.

In addition, virus-specific CTL were generated efficiently from splenocytes obtained from mice 9 days p.i. and the inhibition observed with con A induced IFN-γ (T_H1 cytokine) secretion in i.c.-injected mice was comparatively lower than that of IL-4 (T_H2 cytokine). Intraperitoneal and subcutaneous immunization of BALB/c and C57BL/6 mice with JEV induced virus-specific T_H1 responses (Ramakrishna *et al*., 2003). However, CTL responses may not be protective against JEV infections (Pan *et al*., 2001). IL-2 may not play a dominant role in the expansion and differentiation of antigen-specific CD8 cells, although decreased antigen presentation and co-stimulation leading to decreased IL-2 and IL-4 secretion could inhibit helper T cell responses (Zeng *et al*., 2005; Hinrichs *et al*., 2006). This could accentuate the changes already initiated in the case of i.c. challenge, thereby compromising the ability of the immune system to respond rapidly in an effective manner.

The results discussed above were not apparent from *in vitro* studies of JEV infections. We have shown previously that i.c. but not i.v. infection with JEV caused the upregulation of MHC-I on thymocytes (Kavitha and Manjunath, 2004) and that the effects observed were absent in mice i.c.-infected with inactivated virus or in mice protected by prior immunization. Mice i.c.-injected with inactivated virus could not be routinely used as controls in this study due to ethical considerations. However, i.c.-challenge experiments performed with inactivated virus showed that they did not differ from control non-challenged mice. These effects were not apparent 9 days after i.v-injections with JEV. Longer immunizations would have been inappropriate as controls. Common host genes were activated in the mouse brain by JEV as well as rabies virus and their expression correlated with virus load (Saha and Rangarajan, 2003). Hence, we speculate that these effects were the result of active viral replication within the brain and might not be entirely specific to JEV. Generally, the model using

i.c. challenge with JEV is considered as more stringent and we believe that this could be due to the effects described in this study.

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