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Induction of Japanese encephalitis virus-specific cytotoxic T lymphocytes in humans by poxvirus-based JE vaccine candidates

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Poxvirus-based recombinant Japanese encephalitis (JE) vaccine candidates, NYVAC-JEV and ALVAC-JEV, were examined for their ability to induce JE virus-specific cytotoxic T lymphocytes (CTLs) in a phase I clinical trial. These vaccine candidates encoded the JE virus premembrane (prM), envelope (E) and non-structural 1 (NS1) proteins. The volunteers received subcutaneous inoculations with each of these candidates on days 0 and 28, and blood was drawn 2 days before vaccination and on day 58. Anti-E and anti-NS1 antibodies were elicited in most vaccinees inoculated with NYVAC-JEV and in some vaccinees inoculated with ALVAC-JEV. Peripheral blood mononuclear cells (PBMCs) obtained from approximately one half of vaccines showed positive proliferation in response to stimulation with live JE virus. Cytotoxic assays demonstrated the presence of JE virus-specific CTLs in in vitro*-stimulated PBMCs obtained from two NYVAC-JEV and two ALVAC-JEV vaccinees. Cell depletion tests using PBMCs from one NYVAC-JEV recipient indicated that the phenotype of CTLs was CD8$^+$CD4 .* © *1998 Elsevier Science Ltd. All rights reserved*

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Japanese encephalitis (JE) virus is a medically important flavivirus distributed widely in East, through Southeast and South Asia[1,2]. The only JE vaccine currently approved in most nations for human use is a formalin-inactivated JE virion preparation purified from infected mouse brains (Biken vaccine). This inactivated JE vaccine is efficacious, but is expensive, poses safety risks in production, is occasionally allergenic, and requires three doses. The development of second generation flavivirus vaccines using molecular technologies[3] has been designed to address these concerns and to immunize in one dose, if possible.

A protective role of cytotoxic T lymphocytes (CTLs) against viral diseases has been reported with lymphocytic choriomeningitis[4] and influenza[5]. In JE virus infection, antibody has been considered to play an important role in protection as demonstrated by passive transfer of monoclonal antibodies against the envelope (E) protein[6,7]. On the other hand, a role of cellular immunity in protection has been reported. The adoptive transfer of JE virus-immune T cells protected mice from lethal challenge[8–10]. Mice infected with JE virus developed CTLs that were able to lyse JE virus-infected cells[10,11]. Furthermore, a recent review has questioned whether humoral immunity is the only effective means of providing protection against flavivirus infection[12]. These reports suggested that, in addition to antibodies, CTLs are important in protection from JE.

Immune responses against infectious and non-infectious agents differ in some respects. Non-infectious antigens induce mainly specific CD4$^+$T lymphocytes and antibody production, but usually not CD8$^+$ CTLs. Infectious viruses tend to induce specific CD8$^+$ T lymphocytes including CTLs[13]. Therefore, it is

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likely that inactivated vaccines, like the Biken vaccine, induce predominantly $CD4^+$ T lymphocytes and humoral immunity in humans.

We have developed poxvirus-vectored recombinant JE viruses for use as human vaccines[14,15]. These vaccine candidates contain the premembrane (prM), E and nonstructural 1 (NS1) genes of JE virus in either a highly attenuated vaccinia virus strain (NYVAC) or a canarypox isolate (ALVAC)[16]. Immunization of outbred mice with these candidates induced neutralizing (NEUT) antibodies and conferred protection against lethal JE virus challenge[14,15]. Furthermore, our recent studies have demonstrated that these vaccine candidates are able to induce JE virus-specific $CD8^+$ CTLs in mice[11]. Following safety and efficacy tests using rhesus monkeys (Vaughn *et al.*, in preparation), these candidates were subjected to a human phase I clinical trial (Kanesa-Thasan *et al.*, in submission). The present study was undertaken to determine whether these poxvirus-vectored JE vaccine candidates induce JE virus-specific $CD8^+$ CTLs in humans.

MATERIALS AND METHODS

Viruses

NYVAC-based and ALVAC-based recombinants encoding the JE virus prM, E, and NS1 genes of the Nakayama strain of JE virus (abbreviated as NYVAC-JEV and ALVAC-JEV, respectively) have been described[14,15]. The expression of the JE virus sequences in both recombinants was under the control of the early/late vaccinia promoter (H6). The Nakayama strain of JE virus[17] was used for preparing antigens for radioimmunoprecipitation (RIP) tests and for stimulating peripheral blood mononuclear cells (PBMCs) in proliferation and cytotoxic assays. Viruses used for infection of target cells in cytotoxic assays were the Copenhagen strain of vaccinia virus (vP410) and a vP410-based recombinant encoding the prM, E and NS1 genes of the Nakayama strain of JE virus (vP555)[18].

Vaccination

A total of 19 male and 11 female adult volunteers were randomly grouped according to immunogens and preimmunity status against vaccinia. All volunteers were negative for neutralization antibody against JE virus. Results of one vaccinee (#11) whose postimmunization samples were not obtained are not included in this paper.

Immunogens were (1) 4×10^6 plaque-forming units (p.f.u.) of NYVAC-JEV, (2) 6×10^5 p.f.u. of ALVAC-JEV, (3) 1 dose of Biken vaccine produced from the Nakayama strain of JE virus, and (4) saline. The infectious titers of recombinant vaccines were based on the assay results of reconstituted inocula, although the titers of one dose prior to lyophilization were 5.8×10^6 p.f.u. for NYVAC-JEV and 3.1×10^6 p.f.u. for ALVAC-JEV. Volunteers received three subcutaneous inoculations on days 0, 7 and 28 with each of these immunogens, except for NYVAC-JEV and ALVAC-JEV recipients who were inoculated with saline in place of vaccines on day 7. Venous blood was drawn from all vaccinees on day −2 (2 days before vaccination) and on day 58, except one (#13) whose blood was collected on day 44.

Human plasma and PBMC samples

PBMCs were separated from blood by Ficoll–Hypaque density gradient centrifugation as described[19], and resuspended at a concentration of 1×10^7 ml^{-1} in RPMI1640 medium containing 10% fetal bovine serum and 10% dimethyl sulphoxide. These PBMCs were cryopreserved in liquid nitrogen until use for proliferation and cytotoxic assays. Plasma samples obtained during PBMC preparation were kept frozen until use for RIP tests.

Radioimmunoprecipitation (RIP)

Plasma specimens were tested for their ability to immunoprecipitate ^{35}[S]-labeled viral proteins harvested from the culture fluid of JE virus-infected cells as described[20].

Proliferation assay of PBMCs

The viral antigen used in proliferation assays was live JE virus, which was prepared as previously described[21]. The culture fluid of infected C6/36 cells containing JE virus at approximately 2×10^8 p.f.u.per ml was used as JE viral antigen. The control antigen was culture fluid of mock-infected C6/36 cells.

Proliferative responses of PBMCs were measured as previously described[21]. Briefly, 2×10^5 PBMCs were cultured with each of the viral and control antigens at 1:4, 1:8 and 1:16 dilutions in 200 μl AIM/V medium (Gibco BRL, Gaithersburg, MD) containing 10% human AB serum in 96-well V-bottom microplates at 37°C. Six days after antigen addition, cells were pulsed with 1 μCi of tritiated ([^{3}H]) thymidine for 18 h before being harvested using a multiharvester (Titertek, Skatron Inc., Sterling, VA), and [^{3}H]thymidine incorporation was determined by a liquid scintillation counter (1205 Betaplate; Pharmacia, Wallac Oy, Finland). Stimulation indices were calculated by the standard formula: counts per minute (c.p.m.) induced by JE antigen/c.p.m. induced by control antigen at the same dilution. The criteria to determine positive responses were; (i) a stimulation index equal to or greater than 2.7[21], (ii) a significant difference between c.p.m. induced by the viral and control antigens as evaluated by the Student *t*-test, and (iii) a total c.p.m. greater than 1500 obtained with viral antigens. Proliferative responses were defined as positive if all of these criteria were fulfilled in at least one dilution of stimulating antigens.

Cytotoxic assays

The target cells used for this assay system were autologous Epstein Barr virus-transformed lymphoblastoid cell lines (LCLs) infected with vP555 or vP410 at an m.o.i. of 10 p.f.u./cell or mock-infected 15–20 h before the assay. LCLs were prepared from PBMCs of each individual as described[22]. Under these conditions, the proportion of target cells expressing viral antigens was usually 40–60% and occasionally higher as deter-

mined by an indirect fluorescent antibody assay using a monoclonal antibody to the JE virus E protein (J3-11B9)[18]. LCLs were resistant to infection with JE virus: only 2–3% of the cells were JE antigen-positive even after infection at a high m.o.i.

Cytotoxic activities were measured as previously reported[23]. PBMCs ($4–5 \times 10^6$) were stimulated by incubation with live JE virus at a final dilution of 1:8 in 2 ml of AIM/V containing 10% human AB serum per well of 24-well microplates at 37°C for 7–9 days. These cells were washed three times with RPMI–10%FBS and distributed in microplates at different cell densities to provide various effector:target (E:T) ratios. The target cells were labeled with $Na^{51}CrO_4$, washed three times with RPMI–10%FBS, counted for viable cells, and distributed at 1×10^3 cells in 100 μl per well into microplates containing effector cells. The plates were incubated for 5 h at 37°C, and ^{51}Cr release into the supernatant was measured using a gamma counter. Percent specific lysis was calculated by a formula: $100 \times$ (experimental release − minimum release)/(maximum release − minimum release), where the maximum release was obtained by lysing all the target cells with polyoxyethylene (12) tridecyl ether (Renex; Ruger Chemical Co.,Irvington, NJ) at a final dilution of 1:40 and the minimum release was obtained with target cells incubated alone in RPMI–10%FBS.

Phenotypic analysis

Cell depletion tests were performed using anti-CD4 (OKT4), anti-CD8 (OKT8; Ortho Diagnostic Systems, Inc., Raritan, NJ) and anti-CD16 (anti-natural killer (NK) cells) antibodies (Leu11b; Becton Dickinson, Mountain View, CA) as previously described[21]. Briefly, approximately 1×10^6 PBMCs stimulated with live virus at a 1:8 dilution for 7 days were incubated with each antibody at a 1:11 dilution at 4°C for 30 min and then treated with rabbit complement (Cedarlane Laboratories, Ontario, Canada) at a 1:5 dilution at 37°C for 1 h. These cells were used in cytotoxic assays at an E:T ratio of 75:1.

RESULTS

Elicitation of antibody determined by RIP

Plasma samples obtained from vaccinees before and after vaccination were examined by RIP for antibodies against E and NS1 which were both encoded by candidate vaccines (*Figure 1*, see also *Table 2* for summary). Although volunteers were originally screened for the absence of NEUT antibody and of travel history to any JE-endemic area before immunization, RIP detected anti-E antibody in volunteers #05, #26, #13, #35, #07, #16, #36 and #41 at various levels on day −2, probably because of crossreactive non-neutralizing antibody to JE virus produced by a prior yellow fever (YF) vaccination. Most of these reactivities were only detectable with prolonged exposure of the gels used to produce the autoradiographs shown in *Figure 1*.

The overall intensity of the bands on day 58 was strong in volunteers immunized with NYVAC-JEV and Biken vaccine, and less strong in ALVAC-JEV recipients. Anti-E antibody was raised in all recipients of Biken vaccine, and anti-E and/or anti-NS1 antibodies were raised in most recipients of NYVAC-JEV and some recipients of ALVAC-JEV. In saline recipients, intensity of the reaction was not different between days −2 and 58. These results demonstrate that recombinant JE vaccine candidates have an ability to induce antibodies to E and NS1 in humans.

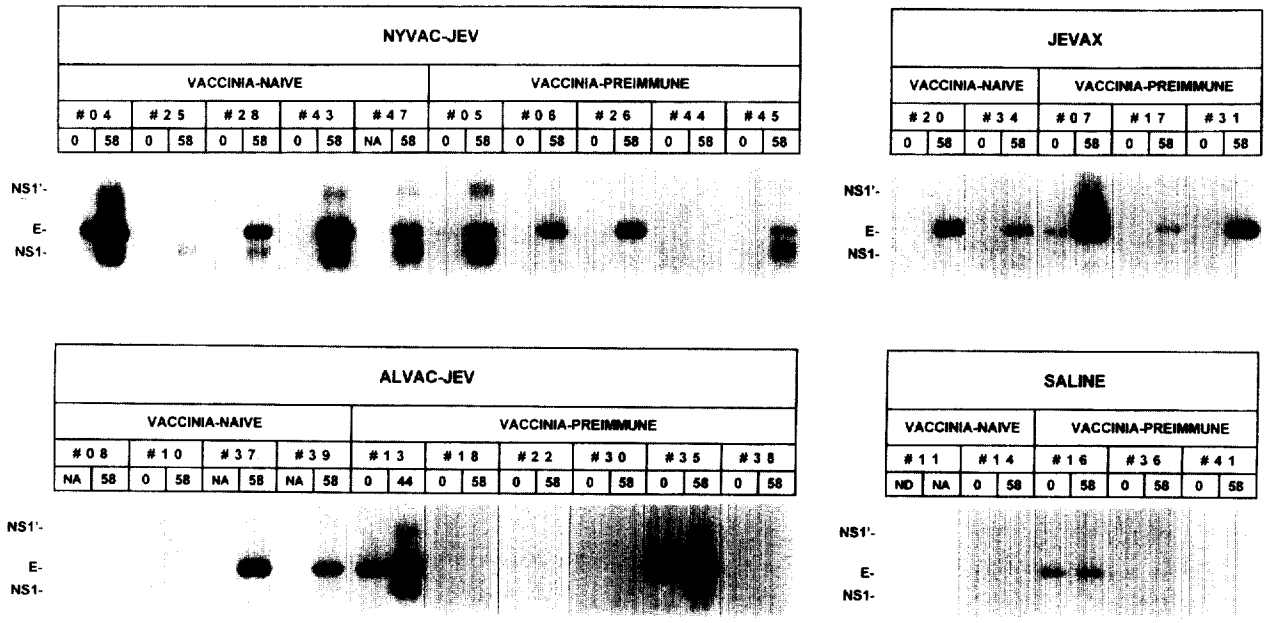

Figure 1 JE virus-specific reactivity of plasma samples obtained from vaccinees who received inoculations with NYVAC-JEV, ALVAC-JEV, Biken vaccine (JEVAX) or saline. Vaccinees were grouped according to vaccinia preimmunity, and each vaccinee is represented by a code number. Plasma samples were collected 2 days before vaccination (represented as day 0) and on day 58 from all vaccinees except #13 whose plasma was collected on day 44. These specimens were tested for their ability to immunoprecipitate radiolabeled proteins harvested from JE virus-infected cells. The positions of JE virus proteins are shown at the left side of the autoradiogram: E, envelope protein; NS1, non-structural protein 1; NS1′, a higher molecular weight form of NS1 produced by alternative processing of the sequences encoded by the NS2A region of the JE virus genome.

All four NYVAC-JEV recipients who were vaccinia-naive and had no detectable antibodies to JE virus E and NS1 on day −2, raised antibodies to both E and NS1 by day 58, whereas one of the three vaccinia-preimmune volunteers did not elicit antibodies to these JE viral proteins. The levels of antibody to E elicited in vaccinia-preimmune vaccinees was slightly lower than those in vaccinia-naive vaccinees. These results suggest that preimmunity against vaccinia may have an adverse

Table 1 Proliferative responses of human PBMCs to live JE virus

			Stimulation index (c.p.m.)[a]					
	Vaccinia		Prevaccination[b]			Postvaccination[c]		
Immunogen	Preimmunity	Code	1:4[d]	1:8	1:16	1:4	1:8	1:16
NYVAC-JEV	–	#04	2.2 (5739)	0.8 (2267)	0.8 (1118)	*3.7* (25482)	*2.7* (21688)	*3.5* (13991)
		#25	1.6 (1329)	0.3 (156)	1.2 (371)	1.7 (1997)	0.3 (172)	1.6 (217)
		#28	1.8 (764)	3.7 (1236)	1.4 (476)	*6.0* (3703)	3.5 (1992)	4.6 (1704)
		#43	1.9 (2480)	1.2 (542)	5.5 (1984)	3.7 (3661)	3.6 (1345)	2.0 (594)
		#47		NA[e]		*3.3* (23838)	2.2 (13973)	2.5 (13840)
NYVAC-JEV	+	#05	2.4 (3552)	0.8 (1486)	2.4 (973)	1.6 (8681)	1.5 (6060)	1.7 (5480)
		#06	5.0 (1992)	5.2 (1229)	1.3 (707)	1.7 (25025)	1.8 (21671)	1.3 (12585)
		#26	1 4 (348)	1.4 (194)	1.1 (166)	1.7 (6219)	0.9 (2161)	0.8 (2228)
		#44	1.0 (1785)	4.2 (1896)	3.9 (1210)	*4.5* (7914)	*5.7* (3973)	0.3 (1005)
		#45	2.0 (4656)	0.2 (1220)	1.5 (1083)	2.1 (17338)	1.4 (9343)	3.6 (9637)
ALVAC-JEV	–	#08		NA		1.6 (100)	1.1 (61)	1.2 (83)
		#10	8.0 (1236)	1.1 (321)	0.6 (221)	1.9 (4292)	*6.9* (5901)	1.7 (2150)
		#37		NA		1.2 (5235)	*7.9* (3428)	0.5 (1291)
		#39		NA		0.9 (16654)	1.1 (10979)	0.7 (5317)
ALVAC-JEV	+	#13	1.6 (1208)	0.6 (200)	2.8 (850)	0.5 (6019)	0.5 (4546)	0.7 (4287)
		#18	1.1 (1035)	1.8 (2491)	2.6 (2340)	1.3 (24935)	1.1 (11910)	1.1 (11241)
		#22	0.3 (567)	0.4 (584)	0.6 (262)	2.7 (1360)	*2.7* (2742)	0.3 (358)
		#30	0.8 (686)	2.6 (718)	1.0 (407)	*3.0* (12947)	2.2 (3472)	1.4 (3738)
		#35	2.4 (19835)	1.2 (7398)	*3.3* (10016)	1.5 (10940)	*3.5* (11997)	*4.6* (4171)
		#38	0.7 (1281)	0.8 (1379)	8.0 (942)	4.0 (4004)	1.2 (2350)	1.5 (1718)
JEVAX	–	#20	0.2 (248)	0.5 (196)	0.6 (142)	1.7 (5323)	0.6 (1851)	0.9 (3175)
		#34	1.3 (662)	1.7 (281)	0.7 (225)	1.8 (13709)	1.0 (5931)	1.6 (8194)
JEVAX	+	#07	1.0 (16321)	1.2 (15663)	0.6 (6693)	1.0 (38350)	0.9 (28001)	1.4 (28323)
		#17	0.9 (9244)	0.6 (7148)	0.9 (3271)	0.7 (2889)	0.2 (826)	0.2 (299)
		#31	2.0 (148)	1.3 (227)	0.8 (94)	1.5 (3786)	0.8 (1791)	2.6 (2472)
Saline	–	#14	2.7 (588)	1.8 (84)	2.9 (435)	5.9 (3593)	2.0 (1658)	3.3 (623)
Saline	+	#16	2.2 (2709)	1.0 (1099)	0.4 (1052)	1.9 (7082)	1.1 (4308)	1.2 (4556)
		#36	6.6 (2501)	3.6 (671)	0.5 (307)	1.6 (342)	0.8 (185)	1.4 (218)
		#41	2.1 (3525)	0.5 (1444)	1.7 (1164)	*3.3* (17028)	2.0 (11990)	1.4 (4852)

[a]Underlined values indicate significant levels of proliferation (see Materials and Methods). Values in parentheses are c.p.m. obtained by stimulation with viral antigens. The data represent the averages of three wells.
[b]Samples collected prevaccination on day −2
[c]Samples collected on day 58 except for the #13 sample which was collected on day 44
[d]Dilution of stimulating antigen (JE virus)
[e]Not available

effect on induction of JE virus-specific humoral immune responses by NYVAC-JEV.

Proliferative responses of PBMCs

To study cellular immune responses in vaccinees, PBMCs obtained from vaccinees before and after vaccination were examined for proliferative responses against live JE virus (*Table 1*, see also *Table 2* for summary). Prevaccination samples were all negative for proliferative responses, except one from vaccinee #35 who had a high level of crossreactive anti-E antibody prior to vaccination. One saline recipient (#41) who showed positive proliferative responses on day 58 was weakly positive for antibody on day −2. None of the Biken vaccinees had detectable proliferative responses on day 58. In the NYVAC-JEV vaccination group, 2 of 4 vaccinia-naive volunteers who were negative for anti-E prior to vaccination had positive proliferative responses on day 58, but only 1 of 5 vaccinia-preimmune volunteers, including those who had crossreactive anti-E antibody prior to vaccination, had positive responses. This difference was not significant ($0.3 < P < 0.5$ by the Chi-square test).

In the ALVAC-JEV vaccination group, 2 of 4 vaccinia-preimmune volunteers who were negative for anti-E prior to vaccination had positive proliferative responses after vaccination. The only vaccinia-naive volunteer whose blood sample was available on day −2 and was negative for anti-E had positive proliferative

Table 2 Summary of immune responses against JE virus antigens

Immunogen	Vaccinia Preimmunity	Code	Anti-E[a]		Anti-NS1[a]		Proliferation[b]		CTL[c]
			Pre[d]	Post[d]	Pre	Post	Pre	Post	Post
NYVAC-JEV	–	#04	–	+	–	+	–	+	+
		#25	–	+	–	+	–	–	ND[e]
		#28	–	+	–	+	–	+	–
		#43	–	+	–	+	–		+
		#47	NA[f]	+	NA	+	NA	+	ND
				(4/4)[g]		(4/4)[h]		(2/4)[i]	(2/3)[j]
NYVAC-JEV	+	#05	+	+	–	+	–	–	ND
		#06	–	+	–	–	–		ND
		#26	±[k]	+	–	–	–		ND
		#44	–	–	–	–	–	+	–
		#45	–	+	–	+	–	–	–
				(2/3)[g]		(2/5)[h]		(1/3)[i]	(0/2)[j]
ALVAC-JEV	–	#08	NA	–	NA	–	NA		ND
		#10	–	–	–	–		+	–
		#37	NA	+	NA	–	NA	+	ND
		#39	NA	+	NA	–	NA	–	ND
				(0/1)[g]		(0/1)[h]		(1/1)[i]	(0/1)[j]
ALVAC-JEV	+	#13	+	+	–	+	–	–	ND
		#18	–	–	–	–	–	–	ND
		#22	–	±	–	–	–	+	–
		#30	–	–	–	–	–	+	ND
		#35	+	+	–	+	+	+	+
		#38	–	–	–	–	–	–	+
				(1/4)[g]		(2/6)[h]		(2/4)[i]	(2/3)[j]
JEVAX	–	#20	–	+	–	–	–	–	ND
		#34	–	+	–	–	–	–	ND
				(2/2)[g]		(0/2)[h]		(0/2)[i]	(0/0)[j]
JEVAX	+	#07	+	+	–	–	–	–	ND
		#17	–	+	–	–	–	–	ND
		#31		+	–	–	–	–	ND
				(2/2)[g]		(0/3)[h]		(0/2)[i]	(0/0)[j]
Saline	–	#14	–	–	–	–	–	–	–
				(0/1)[g]		(0/1)[h]		(0/1)[i]	(0/1)[j]
Saline	+	#16	+	+	–	–	–	–	ND
		#36	±	±	–	–	–	–	ND
		#41	±	±	–	–	–	+	–
				(0/0)[g]		(0/3)[h]		(0/0)[i]	(0/1)[j]

[a]Antibodies as determined by RIP (see *Figure 1*)
[b]Proliferative responses of PBMCs against JE virus (see *Table 1*)
[c]Cytotoxic activity against vP555-infected autologous LCLs of PBMCs which proliferated in response to stimulation with JE virus (see *Figure 2*)
[d]Prevaccination samples (Pre) collected 2 days before immunization and postvaccination samples (Post) collected on day 58 except for the #13 sample which was collected on day 44
[e]Not determined
[f]Not available
[g]Number of postvaccination samples positive for anti-E antibody/number of prevaccination samples tested which were negative for anti-E antibody
[h]Number of postvaccination samples positive for anti-NS1 antibody/number of prevaccination samples tested which were negative for anti-NS1 antibody
[i]Number of postvaccination samples positive for proliferative responses against live JE virus/number of prevaccination samples tested which were negative for anti-E antibody
[j]Number of postvaccination samples positive for cytotoxic activities/number of samples tested
[k]Only visible with prolonged exposure of the gel

responses on day 58. These results demonstrate that immunization of some of the subjects with recombinant JE vaccine candidates induced JE virus-specific T lymphocytes that proliferated after stimulation with live JE virus *in vitro*.

Cytotoxic activity

The PBMCs, which showed stimulation indices equal to or greater than 2.7 on day 58 samples in proliferation assays, were examined for cytotoxic activity against autologous LCLs infected with vP555. Samples of donors #47 and #37 lacking day −2 PBMCs and the sample of donor #30 with only a small amount of cells available were excluded (see *Table 2*). Four of the 11 PBMC samples examined showed JE virus-specific cytotoxic activities. Two of the four PBMC samples were obtained from vaccinia-naive volunteers vaccinated with NYVAC-JEV and the other two were obtained from vaccinia-preimmune volunteers vaccinated with ALVAC-JEV. The CTL results obtained with the JE virus-stimulated PBMCs of these four vaccinees at an E:T ratio of 100:1 are shown in *Figure 2*. Similar levels of cytotoxic activities were also observed in PBMC samples from the same vaccinees, after being stimulated twice with live JE virus *in vitro* (data not shown). The day −2 sample of vaccinee #04 did not show cytotoxic activities after stimulation with live JE virus (data not shown). These results demonstrate that immunization with the recombinant vaccine candidates induced JE virus-specific memory cytotoxic cells in some of the vaccinated individuals. One of these four vaccinees shown in *Figure 2* (#35) had anti-E antibody and positive proliferative responses against live virus on day −2, but the authors did not examine

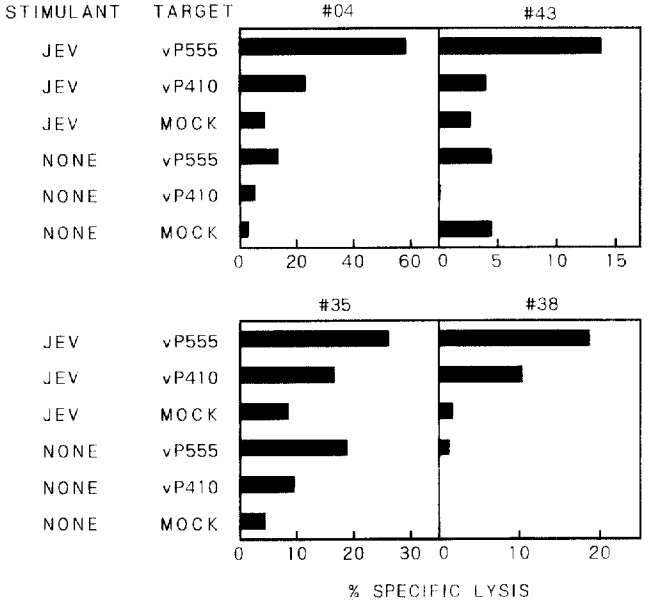

Figure 2 Lysis of LCLs infected with a recombinant encoding the prM, E and NS1 genes (vP555) or the parent vector virus (vP410), or uninfected (MOCK). PBMCs obtained from vaccinees #04, #43, #35 and #38 on day 58 were stimulated with a 1:8 dilution of live JE virus at 37°C for 7–9 days (JEV) or unstimulated (NONE). Cytotoxic activity was measured at an E:T ratio of 100:1 using ^{51}Cr as a marker of cell lysis (see Materials and Methods for details).

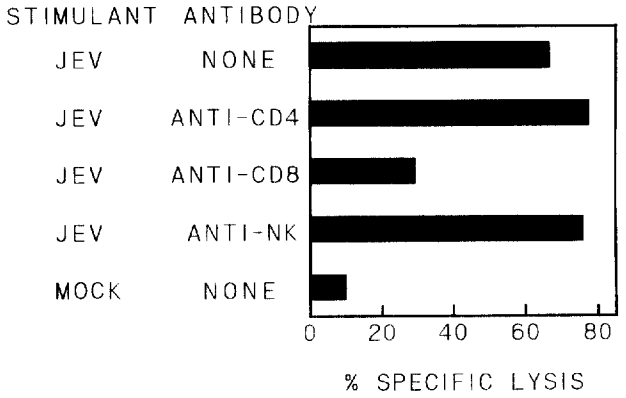

Figure 3 Phenotypic analysis of CTLs by cell depletion using indicated antibodies and complement. PBMCs obtained from vaccinee #04 (vaccinia-naive, NYVAC-JEV-vaccinated) on day 58 were stimulated with a 1:8 dilution of live JE virus for 7 days (JEV) or unstimulated (MOCK). After cell depletion (see Materials and Methods for details), cytotoxic activities were measured using ^{51}Cr-labeled autologous LCLs infected with vP555 at an E:T ratio of 75:1. 'NONE' indicates treatment of cells only with complement.

whether cytotoxic activity was present in the day −2 PBMCs.

In order to characterize the cytotoxic cells, we depleted specific T lymphocyte populations from the JE virus-stimulated PBMCs using monoclonal antibodies and complement before the cytotoxic assay. The PBMC sample obtained from a vaccinia-naive vaccinee who received NYVAC-JEV vaccination (#04) was used in the experiment (*Figure 3*), since this sample showed the highest cytotoxic activity among the four samples shown in *Figure 2*. The cytotoxic activity against vP555-infected target cells was greatly reduced by treatment with anti-CD8 and complement, compared to the activity after treatment only with complement. On the other hand, reduction in cytotoxicity was not observed after treatment with anti-CD4 or anti-CD16 (anti-NK) in the presence of complement. These results demonstrate that cells responsible for lysis of JE virus-infected LCLs were $CD8^+CD4^-$ T-lymphocytes.

DISCUSSION

The present report demonstrates that poxvirus-based recombinant JE vaccines, NYVAC-JEV and ALVAC-JEV, have the ability to induce JE virus-specific $CD8^+$ memory CTLs in some of the vaccinated subjects. The results are summarized in *Table 2*. In our cytotoxicity assays, vP555 was used for preparing target LCLs, since these cells were resistant to infection with JE virus. Our earlier studies indicated that recombinant JE viral proteins produced in eukaryotic cells infected with vP555 were equivalent to authentic proteins[18,20]. Furthermore, vP555-infected P815 mastocytoma cells served as targets for murine CTLs, and the levels of specific lysis were similar between vP555-infected P815 cells and JE virus-infected primary kidney cells as target cells[11].

Although similar levels of anti-E antibody were induced in Biken recipients and vaccinia-naive, NYVAC-JEV recipients in RIP tests, Biken recipients

had significantly higher NEUT antibody titers (a geometric mean titer of 1:211) than NYVAC-JEV recipients (a geometric mean titer of 1:61)(Kanesa-Thasan *et al.*, in submission): the difference between results of RIP and NEUT tests is due to the ability of RIP to detect both neutralizing and non-neutralizing antibodies. Only one of the 10 ALVAC-JEV recipients developed detectable NEUT antibody. These results indicate that Biken vaccine elicited higher levels of NEUT antibodies than recombinant vaccine candidates at the present immunization doses. However, infectious recombinant vaccines were able to induce JE virus-specific memory CTLs, whereas the non-infectious Biken vaccine did not induce enough memory T lymphocytes to show positive proliferative responses of PBMCs to live JE virus in our tests.

Humoral immune responses induced by ALVAC-JEV were lower than those induced by NYVAC-JEV. The difference in humoral immune responses may be due to the difference in inoculation doses (4×10^6 p.f.u. for NYVAC-JEV and 6×10^5 p.f.u. for ALVAC-JEV). However, lower NEUT titers were also observed in mice immunized with ALVAC-JEV than in those immunized with NYVAC-JEV, although the inoculation dose was identical[11]. Thus, these differences are probably due to different characteristics of the vectors, since the same cassette containing the promoter and JE virus genes was used in both recombinant viruses. It is of interest that both recombinants were able to induce CTLs to similar levels in mice[11] and in some vaccinated humans.

Humoral immune responses induced by immunization with NYVAC-JEV seem to be lower in the vaccinia-preimmune group than in the vaccinia-naive group as determined by RIP. Furthermore, results of NEUT tests using samples from NYVAC-JEV recipients (Kanesa-Thasan *et al.*, in submission) indicated a significant difference in positivity between the vaccinia-naive group (5 positive of 5 examined) and the vaccinia-preimmune group (0 positive of 5 examined). The effect of preimmunity against vaccinia on immunogenicity of vaccinia-based recombinant viruses has also been reported by others[24-26]. Although the *in vivo* events occurring in vaccinated hosts were not investigated in our study, this difference may be due to the vaccinia virus-specific memory CTLs probably present in vaccinia-preimmune volunteers[27,28]. These CTLs may destroy NYVAC-infected cells before JE virus-specific antibody responses are induced to the levels that are attained in vaccinia-naive NYVAC-JEV recipients.

In clinical trials of other recombinant vaccine candidates, the induction of CTLs has been reported with vaccine candidates for human immunodeficiency virus type 1 (HIV-1) vectored by ALVAC[29-31] and by vaccinia virus[32] and for human papilloma virus types 16 and 18 vectored by vaccinia virus[33]. Immunogenicity of ALVAC-JEV in humans observed in the present study seems somewhat lower than that of another experimental ALVAC vaccine against rabies[34]. The dose and inoculation route may be critical factors in the differences in immunogenicity determined in these vaccine trials.

In addition to recombinant virus-based vaccines, there have been reports of successful induction of CTLs in animals by naked plasmid DNA[35] peptides administered with adjuvants[36,37] or liposomes[38], and peptides expressed in particulate carriers[39]. The analysis of memory T lymphocytes and antibodies will help us to evaluate new types of preventive and therapeutic vaccine candidates for flaviviruses.

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