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PRINCIPAL INVESTIGATOR: Francis A. Ennis, M.D.

CONTRACTING ORGANIZATION: University of Massachusetts
Medical Center
Worcester, Massachusetts 01655

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Francis A. Ennis, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Massachusetts Medical Center
Worcester, Massachusetts 01655

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Members of the U.S. Armed Forces receive immunization with vaccinia virus vaccine. Efforts to develop a safe and effective tissue culture-derived vaccinia vaccine to replace the standard vaccine produced by scarification on cows' skin have advanced early clinical studies. It is generally accepted that protection induced by the conventional vaccinia vaccine correlated with cellular immune responses to live experimental and standard vaccinia vaccines.

During the two years of this contract we have: 1) obtained, separated and cryopreserved peripheral blood mononuclear cells from 92 vaccinees in a clinical study to compare the standard and an experimental small pox vaccine, 2) prepared live and killed vaccinia virus antigens, 3) performed lymphocyte proliferation and cytotoxic T cell assays and 4) performed immunoblot assays on vaccine donor samples. Both vaccines stimulated vaccinia virus-specific humoral and cellular immune responses in the vaccinees, however, the standard smallpox vaccine stimulated significantly stronger immune responses through the traditional cutaneous route of inoculation.

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Francis A. Ennis 12/19/96

PI - Signature Date

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INTRODUCTION

There is a great deal of interest in the potential use of vaccinia virus as a vector for recombinant vaccines against human and veterinary diseases. Despite the success of vaccination in the eradication of smallpox and evidence that cellular immune mechanisms are essential for virus elimination, little is known about cellular immune responses to vaccinia virus. In addition, vaccinia virus-specific, HLA-restricted cytotoxic T lymphocytes (CTL) had not been demonstrated in man (1,2) although virus-specific CTL have been detected to many other human viruses. Studies with murine models have demonstrated the presence of vaccinia virus-specific CD8+ CTL responses that are MHC class I-restricted (3,4). Studies in different animal species demonstrate that these virus-specific CTL responses correlate with recovery from pox virus infections (1,3,5-6), but did assess the potential effector contribution of CD4+ CTL as part of the recovery process. Recently our laboratory described for the first time human vaccinia specific CD4+ and CD8+ CTL responses (7,8), and have further demonstrated that the vaccinia virus-specific T cell memory response is very long lived and could be detected in vitro in PMBC as long as 50 years after primary immunization (14).

In man, the significance of vaccinia-specific cell-mediated immune responses has been demonstrated by studies of vaccinated children who had thymic aplasia. Some of these children died of progressive vaccinia after accidental immunization despite producing anti-vaccinia antibodies and treatment with massive doses of vaccine immune globulin (9,10). Individuals with profound T cell defects associated with Wiscott-Aldrich syndrome also developed disseminated infection after vaccination with vaccinia virus (11). These and other reports (12,13) indicate that antibody production is not sufficient in protecting man from the complications of smallpox vaccination.

Certain members of the US Armed forces may require smallpox immunizations during future deployments. In addition, the Centers for Disease Control (CDC) has recommended that laboratory personnel involved in research which utilizes vaccinia virus or any of the various recombinant viruses be vaccinated. Only a limited amount of the licensed Wyeth smallpox vaccine (DryVAX^{R}) remains available.

There is a desire to produce a modern tissue-culture derived vaccine, to replace the standard vaccine produced by the old methods of scarification of the skin of cows. Efforts to develop a safe and effective modern tissue culture-derived vaccinia vaccine have advanced to the point of early clinical studies. In addition to the need for safe and effective alternative to the current vaccinia vaccine, the new vaccinia vaccine is likely to be used as a vector carrying gene(s) encoding for foreign antigens to immunize against other pathogenic agents with vaccinia as a safe and potent carrier.

It is generally accepted that protection against smallpox induced by the conventional vaccinia vaccine correlated with restriction of lesion size to skin challenge with vaccinia virus vaccine. Therefore, it is important to be certain that newly developed vaccinia vaccines induce cellular immunity.

Due to the risks associated with the traditional method of administration of the small pox vaccine, cutaneous inoculation, this study was designed to evaluate the cellular immune responses generated as result of alternative routes of immunization using the new cell-cultured vaccine.

BODY

We have completed all of the experiments that were scheduled for year 1 and option year 1 as outlined in the original statement of work. A description of the experiments and results are outlined below:

YEAR 1

1. Preparation of inactivated vaccinia virus antigens

2. Preparation of live vaccinia virus pools

Plates of CV1 cells were infected with vaccinia virus (NYCBH) at a MOI of 10:1 for 2 hours and media was added and plates were incubated overnight. Plates containing control CV1 cells not infected with vaccinia virus were also established. The next day the vaccinia virus infected plates demonstrated cytopathic effects (CPE). Supernatant fluids were discarded and the cells were removed by scraping, resuspended in medium, sonicated and pelletted and aliquots of the remaining supernatant were frozen at -70°C to be used as live virus. The titer of this live vaccinia virus pool is $6.3x10^8$ PFU/ml Aliquots of supernatants from similarly treated infected and control cells were boiled for 10 min and then frozen at -70° to be used as inactivated virus and control antigens. Boiling the virus was more effective than UV-treatment for inactivation. The boiled aliquot from the virus infected supernatant was later tested for residual infective virus on CV1 cells and no CPE was detected.

3. Establishment of vaccinia virus-specific T cell assays

Proliferation in bulk culture

The procedures used have been described in detail in the original grant proposal. Briefly, PBMC was separated on the day of blood drawing and cryopreserved in liquid nitrogen until tested. Thawed PBMC were stimulated with predetermined concentrations of vaccinia antigens and cultured in RPMI media containing 10% heat-inactivated human AB serum. On day 5, the cells were pulsed with ^{3}H-thymidine and incorporation into DNA was measured on day 6. Four replicates were tested with each concentration, and controls included an antigen prepared from uninfected cells or no antigen. The PBMC of a known high responder was also stimulated with UV inactivated virus or live virus as a positive control in each experiment. Antigens were pretested with the PBMC of a non-immunized donor in preliminary experiments to rule out non-specific cell stimulation. Statistical analysis: Briefly we have defined as potentially significant vaccinia memory proliferation responses (I) a $\geq$ 2-fold specific increase above the CPM of the day 0 PBMC, we compared these two groups by Student's t-test. In samples that have a $\geq$ 2 fold increase in CPM in 6 week (1 yr, 3 yr) over CPM stimulated with control antigen we also

compared these groups of CPM by Student's t-test.

Cytotoxic T Cell Assays

These assays were performed as described in the original grant and are published (7,8,14). Briefly, UV inactivated antigens and live virus were used to stimulate PBMC for use as effector cells. On day 7 after stimulation, the stimulated effector cells were added to ^{51}Cr labeled autologous EBV-transformed B-LCL target cells infected with vaccinia or to uninfected target cells.

4. Identification of normal vaccinia virus immune donors with high T cell responses to vaccinia virus antigens.

Three healthy adults who had been reimmunized with vaccinia virus approximately three months, three years, or seven years previously, as well as a vaccinia-naive donor, supplied PBMC for us to perform preliminary experiments to gain information about the specificity and sensitivity of our vaccinia specific T cell lymphoproliferation assays. These donors were laboratory workers who were immunized because they were working with vaccinia recombinant viruses and the non-vaccinia immune donor was a laboratory employee who was not working with vaccinia virus. These individuals donated blood as part of an IRB approved protocol at the University of Massachusetts.

Briefly, proliferation assays were performed in which $2x10^5$ PBMC were added per well and replicates of 3 wells each were exposed to:

Control antigen diluted 1:20, 1:100, 1:500, 1:2500
Killed vaccinia antigen diluted 1:20, 1:100, 1:500, 1:2500
Live vaccinia virus at an MOI of 1, 0.5, 0.25, 0.125

The medium used was RPMI +10% heat inactivated human AB serum (ABI). On day 4 we added tritiated thymidine to the wells and incubated for 15 hrs longer. On day 5 the plates were harvested using a Skatron cell harvester and radioactivity was counted in an LKB beta plate reader.

The results shown below in Table 1 demonstrate that the PBMC of all three donors had little lymphoproliferation following exposure to the control CV1 antigen. A very brisk lymphocyte proliferative response was detected using the killed vaccinia antigen with stimulation indices of approximately 100:1 at multiple antigen dilutions for each of the three donors. Somewhat lower but very convincing proliferative responses were also observed using PBMC exposed to the live virus antigen. Because of the availability of a relatively large number of PBMC from the donor who had been immunized 7 years ago and the very convincing proliferative responses to both live virus and inactivated antigen (stimulation indices of over 100:1 and about 45:1 respectively) we decided to use the PBMC of this donor as a positive control in subsequent experiments.

Table 1. Proliferation of human immune PBMC to Vaccinia Antigens

	PBMC of Donors who were revaccinated after					
	3 months		7 years		3 years	
Antigen	CPM	SI	CPM	SI	CPM	SI
Medium	196.96		172.23		592.51	
Live virus (M01)						
1.0	868.9	4.4	1631.9	9.5	23665.0	39.9
0.5	577.4	2.9	7774.5	45.1	27381.3	46.2
0.25	3457.4	17.6	8336.8	48.4	16008.6	27.0
0.125	1912.8	9.7	5535.6	32.1	16836.5	28.4
Killed virus						
1:20	23605.3	119.9	31197.2	181.1	69429.6	117.1
1:100	18814.8	95.5	23151.9	134.4	44460.1	75.0
1:500	10616.7	53.9	17552.0	101.9	30977.8	52.3
1:2500	4881.1	20.7	3628.1	21.1	19833.5	33.5
Control Ag						
1:20	335.3	1.7	812.8	4.7	1183.0	2.0
1:100	354.2	1.8	538.0	3.1	670.9	1.1
1:500	324.4	1.7	358.6	2.1	632.7	1.1
1:2500	311.0	1.6	206.7	1.2	555.3	.9

Following the demonstration of brisk lymphoproliferative responses to both live and inactivated vaccinia antigens but not to the control cell antigen we tested the PBMC of a young adult who had no history of being immunized with vaccinia vaccine. The results in Table 2 demonstrate that the PBMC of the non-vaccinated donor were not stimulated by either the live virus or the inactivated vaccinia antigen preparation. The positive control vaccinia-immune donor's PBMC were stimulated to a significant degree by both the inactivated and live vaccinia virus antigen preparations.

Table 2. Proliferative Response of PBMC for Vaccinia Non-Immune and Immune Donor

	Non-Immune		Immune	
	CPM	SI	CPM	SI
Medium Control	3057.1		354.5	
Live virus (MOI)				
.8	2577.4	0.8	2877.8	8.1
.4	3136.2	1.0	2529.1	7.1
.2	2454.1	0.8	4469.6	12.6
.1	2666.5	0.9	6008.0	16.7
Killed virus Ag				
1:40	15086.0	1.6	25535.3	31.4
1:80	12963.8	1.2	23650.1	25.1
1:160	8800.5	2.0	18552.3	31.6
1:320	10378.4	3.6	18358.7	50.0
Control Ag				
1:40	9399.2		813.3	
1:80	10793.5		940.9	
1:160	4391.0		587.8	
1:320	2917.8		367.1	

5. The acquisition, separation, and cryopreservation of peripheral blood mononuclear cell (PBMC) from paired samples from all vaccinees (92 total volunteers) on day 0 and 4 weeks post inoculation in the phase II clinical trial sponsored by USAMRIID at Fort Sam Houston.

Two of our scientists made 5 trips to Ft. Sam Houston, San Antonio Texas to obtain and process specimens of human PBMC required for testing lymphocyte responses to the experimental vaccinia vaccine being tested in volunteers by the U.S. Army scientists. The number of volunteers whose PBMC were obtained, processed and cryopreserved in liquid nitrogen was 32, 57 and 22 on three pre-vaccine groups, and 31, 47 and 24 in the three post-vaccine groups. In total the number of paired serum samples was 92, because 19 donors did not have a second blood sample obtained.

Briefly, up to 9 vacutainers of CP Tubes (Becton Dickinson Cat #362761) were filled per donor bleed. The tubes were centrifuged at 1500 g x 20 minutes at room temperature in a horizontal rotor (swing-out). The tubes were inverted twice, and caps were removed in a laminar flow hood. Cells and plasma were transferred into 2 x 50 ml centrifuge tubes (Falcon #2070) at 35 ml/tube and were transported back to UMMC. At UMMC in Worcester, MA (the next day

after blood was obtained), the tubes were centrifuged at 400 g x 5 min. Seven mls of plasma/donor/bleed were placed in a vial and stored at -20°C. After the remaining fluid was aspirated, the cells were resuspended in 25 ml PBS pH 7.2 (Gibco Cat. #20012-019) and centrifuged at 300 g x 5 min. Cells were then resuspended in 24 ml of RPMI and were recentrifuged. The cell pellet was then resuspended in 5 ml RPMI, 20% heat inactivated FBS and 10% DMSO. One ml was added/cryovial (Corning #25704), 5 vials/donor/bleed, and were frozen in liquid nitrogen with a rate controlled freezer (Cryomed Model 801).

6. Bulk culture lymphocyte proliferation assays on day 0 and 27 samples from all vaccinees in this trial.

We have completed under code the vaccinia-specific T lymphocyte proliferation responses on all of the volunteers' day 0 and day 27 PBMC samples. A copy of those results was sent to Drs. A. Schmaljohn and McClain who subsequently sent a copy of the vaccine group code to us. We have tabulated the responses according to each vaccine study group and developed an overall summary. Copies of these results are contained in Tables 4-8 and the raw data was sent on computer disc to Dr. A. Schmaljohn at USAMRIID. Individuals in all of the vaccine groups had vaccinia-specific T lymphocyte responses, and the three groups that received the TSI-GSD241 vaccine had similar responses whether the vaccine was given intradermally, with or without an alcohol wipe, or intramuscularly. The standard vaccine given by scarification induced T lymphocyte responses in virtually all of the vaccinees. We measured responses to both infectious vaccinia virus and to inactivated vaccinia virus antigens. The results are summarized in Table 3 and all the data are shown in Tables 4-8.

Table 3. The Percentage of Volunteers Who Developed Vaccinia-Specific Memory T lymphocyte Proliferation Responses* in Ft. Sam Houston Study, 1994

Group	Vaccine	Route	Alcohol wipe	#	Live Virus	Killed Virus
1A	TSI-GSD 241	ID	+	17	41	71
1B	TSI-GSD 241	ID	-	16	50	69
2	TSI-GSD 241	IM		26	54	77
3	Wyeth	SCAR		32	91	100

* A positive proliferation response is defined as a Stimulation Index (S.I) at day 27 that is $\geq$ 2 fold above day 0 and also is SS at level of $p<0.05$. Three volunteers who had a $\geq$ 2 fold increase in CPM but p was not <0.05 to live virus but had ($p<0.001$) to killed virus are considered to be positive responders to virus. The three different vaccine groups contained one each of these type of responders.

OPTION YEAR 1

1. Perform the following assays in no less than 20 volunteers from the Phase II Clinical Study (mentioned above):

TABLE 4 GROUP 1A=TSI-GSD 241 INTRADERMALLLY (5.0 LOGS) WITH ALCOHOL WIPE T CELL PROLIFERATION						PRNT**** 50% PROBIT		IMMUNOBLOT REACTIVITY	
UMMC#	ARMY#	VIRUS	2 FOLD	AG	> 2	PRE	D27	PRE	D27
19	1	5.4	Y **	4.7	Y ***	3	3	-	-
12	17	1.5		8.2	Y **	15	5	-	-
*	20	1.3		1.5		11	44	-	+
21	29	1.2		3.3	Y **	30	41	+	+
31	31	1		3.6	Y **	3	5	-	-
30	43	1		2.3	Y **	3	N/A	-	-
66	253	2.6	Y ***	3.9	Y ***	17	76	-	+
52	261	1.5		1.4		3	3	-	-
72	267	1		1.7		5	5	-	-
58	273	4.4	Y *	15.2	Y ***	5	3	-	-
83	280	1.9		5.8	Y ***	3	198	-	-
35	289	2.7	Y *	1		3	5	-	-
34	300	2.3	Y **	4.7	Y ***	5	25	+	+
78	305	1		2.1	Y **	11	16	-	+
92	313	2.8	Y ***	9.6	Y **	3	35	-	-
100	322	2.3	Y *	2.7	Y ***	3	53	-	+
111	333	0.8		1		3	3	-	-

*=p<.05
**=p<.01
***=p<.001

**** PLAQUE REDUCTION NEUTRALIZATION TITERS OBTAINED FROM DR. D. McCLAIN, USAMRIID

TABLE 5 GROUP 1B=TSI-GSD 241 INTRADERMALLY (5.0 LOGS) WITHOUT ALCOHOL WIPE T CELL PROLIFERATION						PRNT**** 50% PROBIT		IMMUNOBLOT REACTIVITY	
UMMC#	ARMY#	VIRUS	2 FOL	AG	> 2	PRE	D27	PRE	D27
27	4	1.8		1.4		3	51	-	+
4	10	2	Y ***	10.5	Y ***	3	11	-	-
8	15	4.8	Y ***	77.3	Y ***	5	57	-	+
29	22	3.7	Y	17.8	Y ***	5	95	-	+
17	27	4.3	Y **	0.7		5	18	-	-
5	35	1.4		5.1	Y *	3	3	-	-
80	263	1		2.6	Y *	13	3	-	-
63	271	12.7	Y ***	6	Y ***	3	640	-	+
79	281	1.1		1.5		3	5	-	-
87	283	1		3.1	Y *	3	12	-	-
44	292	27.6	Y ***	48.3	Y ***	5	277	-	+
40	304	18.4	Y ***	64	Y ***	3	167	-	+
48	309	8	Y ***	10.8	Y ***	3	347	-	+
96	318	1.5		1.9		3	3	-	-
97	319	1		9.1	Y ***	3	229	-	+
108	330	1.6		1.6		3	5	-	-

*=p<.05
**=p<.01
***=p<.001

**** PLAQUE REDUCTION NEUTRALIZATION TITERS OBTAINED FROM DR. D. McCLAIN, USAMRIID

TABLE 6 GROUP 2=TSI-GSD 241 INTRAMUSCULARLY (5.0 LOG T CELL PROLIFERATION						PRNT**** 50% PROBIT		IMMUNOBLOT REACTIVITY	
UMMC#	ARMY#	VIRUS	2 FOLD	AG	> 2	PRE	D27	PRE	D27
13	5	5	Y	16.3	Y ***	3	3	-	-
3	9	1		1.9		3	3	-	-
26	16	2.5	Y *	1.4		31	46	+	+
7	18	8.2	Y **	3.6	Y ***	109	100	+	+
18	24	1.9		2.1	Y **	3	3	-	-
28	28	5.7	Y **	11.2	Y ***	25	N/A	+	+
15	30	1		3.4	Y ***	3	3	-	-
6	33	1.5		45.3	Y **	6	44	-	-
23	37	9.5	Y ***	5.1	Y ***	94	50	+	+
46	251	23.5	Y ***	25	Y ***	3	128	+	+
39	257	1.8		9.5	Y ***	16	9	-	-
69	260	11.1	Y ***	15.6	Y ***	18	69	-	+
81	264	1.1		6.9	Y ***	3	3	-	-
70	265	1		5.3	Y	3	37	-	-
65	270	5.1	Y ***	1.5		3	5	-	-
55	272	5.2	Y **	5.6	Y ***	5	28	-	-
75	277	2.1	Y **	6.3	Y ***	3	32	-	-
60	282	13.9	Y ***	29.1	Y ***	12	3	-	-
54	287	4.8	Y ***	7.8	Y ***	3	42	-	+
53	288	7.4	Y ***	5.2	Y ***	3	65	-	+
82	290	1.3		8	Y **	5	3	-	-
42	293	5	Y ***	10.5	Y ***	3	5	-	-
45	295	2.5	Y ***	2	Y **	14	5	-	+
68	298	2.7	Y	1.7		5	20	-	-
50	301	0		0		16	17	-	-
77	310	1.4		8.3	Y ***	5	26	-	-

*=p<.05
**=p<.01
***=p<.001

**** PLAQUE REDUCTION NEUTRALIZATION TITERS OBTAINED FROM DR. D. McCLAIN, USAMRIID

TABLE 7 GROUP 3=WYETH DRYVAX BY SCARIFICATION T CELL PROLIFERATION						PRNT**** 50% PROBIT		IMMUNOBLOT REACTIVITY	
UMMC#	ARMY#	VIRUS	2 FOLD	AG	> 2 F	PRE	D27	PRE	D27
24	3	5.5	Y ***	5.4	Y ***	68	256	+	+
11	12	7	Y ***	7.6	Y ***	5	362	-	+
22	13	3.1	Y **	5.4	Y ***	3	137	-	+
16	21	25	Y *	23.9	Y ***	3	63	-	+
10	23	1		2.4	Y *	3	3	-	-
32	25	3.1	Y *	19.8	Y *	20	1516	+	+
2	32	7.8	Y ***	7.9	Y ***	5	727	-	+
9	34	19.8	Y **	45.4	Y ***	3	299	-	+
20	40	2.4	Y	64.1	Y ***	3	97	-	+
25	42	16.8	Y ***	44.7	Y ***	3	114	-	+
64	254	6.1	Y ***	3.4	Y ***	8	68	-	+
85	258	27.7	Y ***	38.9	Y ***	3	286	-	+
84	259	11.3	Y ***	35.8	Y ***	5	712	-	+
61	262	12.8	Y ***	21.7	Y ***	3	416	-	+
86	266	12.9	Y ***	18.8	Y ***	5	227	-	+
49	268	5.4	Y ***	8.9	Y ***	3	162	-	+
57	274	3.6	Y ***	16.8	Y ***	5	104	-	+
59	275	6.6	Y ***	7.8	Y ***	3	70	-	+
76	278	1.7		2.9	Y ***	19	70	-	+
62	279	5.3	Y ***	3.6	Y ***	5	135	-	+
73	294	23.9	Y ***	36.2	Y ***	5	664	-	+
37	297	47.8	Y ***	34.5	Y ***	5	615	-	+
43	299	8.6	Y ***	8.7	Y ***	119	315	+	+
41	302	17.5	Y ***	4	Y ***	3	346	-	+
38	303	16.1	Y ***	23.9	Y ***	7	206	-	+
88	307	3.4	Y *	36.2	Y ***	3	687	-	+
90	311	6.8	Y ***	13.9	Y ***	3	164	-	+
93	314	4	Y ***	4.9	Y ***	3	46	-	+
98	320	2.9	Y ***	3.3	Y ***	3	74	-	+
102	324	2.6	Y **	6.3	Y ***	3	119	-	+
104	326	12.1	Y ***	13.3	Y ***	3	48	-	+
106	328	1.9		4.5	Y ***	3	44	-	+

*=p<.05
**=p<.01
***=p<.001

**** PLAQUE REDUCTION NEUTRALIZATION TITERS OBTAINED FROM DR. D. McCLAIN, USAMRIID

TABLE 8 GROUP SCR=TSI-GSD 241 BY SCARIFICATION T CELL PROLIFERATION						PRNT**** 50% PROBIT		IMMUNOBLOT REACTIVITY	
UMMC#	ARMY#	VIRUS	> 2 FOL	AG	> 2 F	PRE	D27	PRE	D27
94	315	1		2.4	Y**	3	3	-	-
95	316	1		1.5		5	167	-	+

**** PLAQUE REDUCTION NEUTRALIZATION TITERS OBTAINED FROM DR. D. McCLAIN, USAMRIID

a. bulk culture CD4+ CTL assays on samples from day 0 and day 28.
b. bulk culture CD8+ CTL assays on samples from day 0 and day 28

We established EBV-transformed B-cell lines from a total of 23 vaccine recipients that had high T-cell proliferation and neutralizing antibody responses. We analyzed the vaccinia virus-specific cytotoxic T cell activity associated with the PBMC from donors who received the standard Wyeth vaccine and donors who received the tissue culture derived vaccine. The methods used for the CTL analysis were described in detail in the original grant application.

Briefly, cryopreserved vaccine donor PBMC from day 0 and day 27 were thawed and stimulated with either live virus or inactivated antigen and incubated for seven days. On day 7 a CTL assay was performed utilizing autologous BLCL target cells that were: uninfected, vaccinia-antigen pulsed, or infected with vaccinia virus. A vaccinia virus-specific CD4+ CTL clone(JC-EA5) was used as the positive control at an E/T ratio of 10:1, and in some experiment a vaccinia virus specific CD8+ CTL clone (JC-DC7) was also included as a positive control. Effector to target ratios of 80 and 40 to 1 were generally used but in some instances lower E/T ratios were tested because fewer PBMC were available after the T cell proliferation assays which we reported previously. The data obtained from CTL assays performed on the cultures derived from 23 donors are outlined in the charts on pages x to y. The donor and group numbers appear at the top left corner of each chart.

Cultures that demonstrated significant vaccinia specific CTL activity on day 7, and had adequate numbers of cells remaining in the culture, had a complement depletion CTL assay performed on the following day to determine the cell types responsible for the observed CTL activity described in the grant application. Briefly, the remaining cultured cells were split into several tubes and incubated with either anti-CD3, anti-CD4, and anti-CD8 or anti-CD16 monoclonal antibodies. After an additional incubation with complement, the cells were used as effector cells in a CTL assay. After incubation with the monoclonal antibody followed by the addition of complement, the cells that react with the antibody are depleted from that sample. The results of the complement depletion CTL assays appear on the right hand side of the chart on the attached pages.

The CTL assays performed for all of the 23 donors derived cultures yielded reliable data because the spontaneous release of all targets in each assay was <30%. Cultures generated from two Wyeth vaccine donors, Army #40 and Army #42 had poor CTL lytic activity, although the proliferation and PRNT antibody data for these donors suggested a good immune response to the vaccine. When these cultures were established, the viability of the PBMC was poor and this probably contributed to the lack of detectable vaccinia-virus specific CTL activity. Complement depletion assays were performed on cultures derived from 11 of the 23 donors (Army 's 34,62,266,282,292,294,297,302,303,304,326).

There was no convincing vaccinia virus specific CTL lysis observed from any culture derived from PBMC collected on day 0 of the study. This is reasonable because none of the 23 donors had a history of vaccinia vaccination prior to this study. 20 out of 23 donors cultures demonstrated measurable vaccinia virus-specific CTL lytic activity on vaccinia virus infected

targets with generally less recognition and lysis of the vaccinia antigen pulsed targets. This is reasonable because high responders (based on the proliferation experiments) were chosen for the CTL analyses. Only one donor, Army #22 whose T cells demonstrated no recognition of vaccinia virus infected target cells in the CTL assay although the proliferation and PRNT data suggested that there would be a vaccinia virus specific response. This donor's lack of a CTL response despite brisk proliferation and PRNT responses is interested. Could this donor have a predominant CD4+ Th2 rather than a CD4+ Th1 response?

Live vaccinia virus stimulation of the day 27 PMBC was a potent stimulus for generating vaccinia virus specific CTL activity. The cultures derived from donors who received the Wyeth vaccine had higher levels of CTL lytic activity than the CTL activity derived from donors who received the TSI-GSD vaccine regardless of the administration route. Analysis of the CTL data obtained from the cultures derived from donors who received the tissue culture derived vaccine suggested that each route of administration was capable of stimulating a vaccinia-virus specific CTL response in vivo. Complement depletion analysis of cultures that recognized and lysed vaccinia virus infected target cells demonstrated that stimulation with live virus preferentially expanded vaccinia virus-specific CD8+ CTL and that stimulation with inactivated vaccinia virus antigen preferentially gave rise to vaccinia virus specific CD4+ CTL.

ARMY #	1						PROLIF. INDEX			PRNT	
UMMC #	19						VIRUS	AG		DAY 0	DAY 27
GROUP	1A	TSI-GSD INTRADERM WITH ALCOHOL WIPE					5.4	47		3	3
			PERCENT SPECIFIC IMMUNE LYSIS								
			OF ^{51}Cr AUTOLOGOUS BLCL								
		E/T	VAC-VIR	VAC-AG	UNINF						
DAY 0	VIRUS	80	-4.6	-3.9	-2.6						
	VIRUS	40	-5.6	-5.2	-0.7						
	AG	80	-4.2	-4.5	-2.3						
	AG	40	-4.9	-4.5	-1.7						
DAY 27	VIRUS	80	12.9	-0.7	-2.6						
	VIRUS	40	2.1	0.2	-3						
	AG	80	35.9	7.2	-0.5						
	AG	40	42.3	7.1	1.2						
MIN/MAX			28.6	20.4	12.9						
CONTROL											
JC EA4	CD4	10	46	43.6	0.3						
MIN/MAX			21.1	12.5	11.2						

ARMY #	15						PROLIF. INDEX			PRNT	
UMMC #	8						VIRUS	AG		DAY 0	DAY 27
GROUP	1B	TSI-GSD INTRADERM W/OUT ALCOHOL WIPE					4.8	77.3		5	57
			PERCENT SPECIFIC IMMUNE LYSIS								
			OF ^{51}Cr AUTOLOGOUS BLCL								
		E/T	VAC-VIR	VAC-AG	UNINF						
DAY 0	VIRUS	80	-2.5	-2.3	-0.1						
	VIRUS	40	-1.9	-1.5	-0.9						
	AG	80	0.6	-0.3	-0.2						
	AG	40	2	-1.2	-1.6						
DAY 27	VIRUS	80	21	12.8	5.7						
	VIRUS	40	29.6	11	2.1						
	AG	80	32	12.7	1.4						
	AG	40	19.9	11.5	2.8						
MIN/MAX			20.3	15.8	15.6						
CONTROL											
JC EA4	CD4	10	46	43.6	0.3						
MIN/MAX			28.6	20.4	12.9						

ARMY #	22						PROLIF. INDEX			PRNT	
UMMC #	29						VIRUS	AG		DAY 0	DAY 27
GROUP	1B	TSI-GSD INTRADERM W/OUT ALCOHOL WIPE					3.7	17.8		5	95
			PERCENT SPECIFIC IMMUNE LYSIS								
			OF ^{51}Cr AUTOLOGOUS BLCL								
		E/T	VAC-VIR	VAC-AG	UNINF						
DAY 0	VIRUS	80	-6.1	-1.6	-1.3						
	VIRUS	40	-4.3	-1.6	-0.8						
	AG	80	-6.8	-2	-1.3						
	AG	40	-6.6	-1.4	-1						
DAY 27	VIRUS	80	-9.1	-2.7	-0.8						
	VIRUS	40	-8.2	-2	-0.8						
	AG	80	-7.9	-1.8	-0.9						
	AG	40	-6.9	-2.7	-1.2						
MIN/MAX			19.3	11.2	10.3						
CONTROL											
JC DC7	CD8	10	35.9	-1.9	-1.4						
JC EA4	CD4	10	37.8	35.7	-1						
MIN/MAX			15.3	12.9	12.3						

ARMY #	34						PROLIF. INDEX			PRNT		
UMMC #	9						VIRUS	AG		DAY 0	DAY 27	
GROUP	3	WYETH					19.8	45.4		3	299	
			PERCENT SPECIFIC IMMUNE LYSIS									
			OF ^{51}Cr AUTOLOGOUS BLCL									
		E/T	VAC-VIR	VAC-AG	UNINF		DAY 27		E/T	VAC-VIR	UNINF	K562
							VIRUS BULK					
DAY 0	VIRUS	40	-3.2	-5.9	-9.5		NO C'		80	NT	NT	NT
	VIRUS	20	NT	NT	NT		C' ONLY		80	69.4	4.1	18
	AG	40	0	-7	-10		C' + ANTI-CD3		80	6.2	0.6	9.9
	AG	20	0	-5.9	-9.6		C' + ANTI-CD4		80	50.3	3.4	18.6
							C' + ANTI-CD8		80	-1.5	1.5	14.9
DAY 27	VIRUS	80	74.6	9.1	-2.6		C' + ANTI-CD16		80	36.9	2.1	2.1
	VIRUS	40	77.6	4.1	-4.4							
	AG	80	31	14.9	-3.5		ANTIGEN BULK					
	AG	40	29.1	8.7	-5.9		NO C'		80	NT	NT	NT
							C' ONLY		80	8.3	2.6	29.2
MIN/MAX			16.9	20.9	19.6		C' + ANTI-CD3		80	-1.6	0	10
							C' + ANTI-CD4		80	2.2	3.3	25.1
							C' + ANTI-CD8		80	4.7	2.9	19.1
							C' + ANTI-CD16		80	5	2.5	14.4
CONTROL												
JC DC7	CD8	10	63.4	-2.3	-4.6		MIN/MAX			20.5	14.4	10.3
JC EA4	CD4	10	30.7	28.8	-1.2							
MIN/MAX			17.6	15.1	17.2							

ARMY #	40						PROLIF. INDEX			PRNT	
UMMC #	20						VIRUS	AG		DAY 0	DAY 27
GROUP	3	WYETH					2.4	64.1		3	97
			PERCENT SPECIFIC IMMUNE LYSIS								
			OF ^{51}Cr AUTOLOGOUS BLCL								
		E/T	VAC-VIR	VAC-AG	UNINF						
DAY 0	VIRUS	40	-5.4	-2.6	-1.8						
	VIRUS	20	NT	NT	NT						
	AG	40	-6.7	-2.6	-2.5						
	AG	20	NT	NT	NT						
DAY 27	VIRUS	40	-4.8	-2.3	-1.5						
	VIRUS	20	NT	NT	NT						
	AG	40	-2.8	-2.4	-2.6						
	AG	20	NT	NT	NT						
MIN/MAX			20.1	15	15.1						
CONTROL											
JC DC7	CD8	10	50	-0.8	-0.7						
JC EA4	CD4	10	48.2	40.7	-2.3						
MIN/MAX			18	11.1	12						

ARMY #	42						PROLIF. INDEX			PRNT	
UMMC #	25						VIRUS	AG		DAY 0	DAY 27
GROUP	3	WYETH					16.8	44		3	114
			PERCENT SPECIFIC IMMUNE LYSIS								
			OF ^{51}Cr AUTOLOGOUS BLCL								
		E/T	VAC-VIR	VAC-AG	UNINF						
DAY 0	VIRUS	40	5.4	-1.2	-4						
	VIRUS	20	NT	NT	NT						
	AG	40	3	-0.6	-4.2						
	AG	20	NT	NT	NT						
DAY 27	VIRUS	40	0.8	-4.1	-5.9						
	VIRUS	20	NT	NT	NT						
	AG	40	-1.4	-1.7	-4.6						
	AG	20	-0.4	-2.5	-4.8						
MIN/MAX			18.9	12.5	13.7						
CONTROL											
JC DC7	CD8	10	50	-0.8	-0.7						
JC EA4	CD4	10	48.2	40.7	-2.3						
MIN/MAX			18	11.1	12						

ARMY #	251						PROLIF. INDEX			PRNT	
UMMC #	46						VIRUS	AG		DAY 0	DAY 27
GROUP	2	TSI-GSD INTRAMUSCULARLY					23.5	25		3	128
			PERCENT SPECIFIC IMMUNE LYSIS								
			OF ^{51}Cr AUTOLOGOUS BLCL								
		E/T	VAC-VIR	VAC-AG	UNINF						
DAY 0	VIRUS	80	1.9	-2.6	-2.7						
	VIRUS	40	0.3	-0.7	-1.4						
	AG	80	3.5	-2.7	-1.7						
	AG	40	14.2	-2.3	-2.6						
DAY 27	VIRUS	80	71.4	10.4	7.9						
	VIRUS	40	65.3	7.2	3.1						
	AG	80	30.3	-2.1	-1.4						
	AG	40	19.7	-0.5	-0.6						
MIN/MAX			13.6	12.8	12.2						
CONTROL											
JC DC7	CD8	10	NT	NT	NT						
JC EA4	CD4	10	25.4	55.8	-3						
MIN/MAX			10.6	12.6	13.9						

ARMY #	258						PROLIF. INDEX			PRNT	
UMMC #	85						VIRUS	AG		DAY 0	DAY 27
GROUP	3	WYETH					27.7	38.9		3	286
			PERCENT SPECIFIC IMMUNE LYSIS								
			OF ^{51}Cr AUTOLOGOUS BLCL								
		E/T	VAC-VIR	VAC-AG	UNINF						
DAY 0	VIRUS	80	NT	NT	NT						
	VIRUS	40	0.2	-0.3	0.8						
	AG	80	NT	NT	NT						
	AG	40	2.1	-0.5	0.7						
DAY 27	VIRUS	80	35.2	5	5.8						
	VIRUS	40	15.8	4.3	4.1						
	AG	80	42.1	4.5	5.1						
	AG	40	20.4	4.3	4.4						
MIN/MAX			19.1	8.4	8.2						
CONTROL											
JC EA4	CD4	10	68.1	61.4	-0.6						
MIN/MAX			15.4	10.6	11.3						

ARMY #	266						PROLIF. INDEX			PRNT		
UMMC #	86						VIRUS	AG		DAY 0	DAY 27	
GROUP	3	WYETH					12.9	18.8		5	227	
			PERCENT SPECIFIC IMMUNE LYSIS									
			OF ^{51}Cr AUTOLOGOUS BLCL									
		E/T	VAC-VIR	VAC-AG	UNINF		DAY 27		E/T	VAC-VIR	UNINF	K562
							VIRUS BULK					
DAY 0	VIRUS	80	0	-0.1	0.4		NO C'		80	NT	NT	NT
	VIRUS	40	-1.6	0.3	0		C' ONLY		80	52.6	7.2	44.6
	AG	80	-1.8	0.9	0.6		C' + ANTI-CD3		80	23.8	11.1	52.3
	AG	40	-3.2	-0.9	0		C' + ANTI-CD4		80	52.3	7	49.6
							C' + ANTI-CD8		80	17	3.5	31.9
DAY 27	VIRUS	80	78.6	17.1	16.8		C' + ANTI-CD16		80	52.7	8	15.9
	VIRUS	40	67.8	15.9	11							
	AG	80	6.8	2.6	2.1		ANTIGEN BULK					
	AG	40	-1	0.1	1.5		NO C'		80	NT	NT	NT
							C' ONLY		80	NT	NT	NT
MIN/MAX			19.5	8.7	7.6		C' + ANTI-CD3		80	NT	NT	NT
							C' + ANTI-CD4		80	NT	NT	NT
							C' + ANTI-CD8		80	NT	NT	NT
							C' + ANTI-CD16		80	NT	NT	NT
CONTROL												
JC EA4	CD4	10	46.2	62.7	-2.3		MIN/MAX			12.3	9.8	10.2
MIN/MAX			16.1	14.7	15.8							

ARMY #	271						PROLIF. INDEX			PRNT	
UMMC #	63						VIRUS	AG		DAY 0	DAY 27
GROUP	1B	TSI-GSD INTRADERM W/OUT ALCOHOL WIPE					12.7	6		3	640
			PERCENT SPECIFIC IMMUNE LYSIS								
			OF ^{51}Cr AUTOLOGOUS BLCL								
		E/T	VAC-VIR	VAC-AG	UNINF						
DAY 0	VIRUS	80	0.3	-2.5	0.4						
	VIRUS	40	-5.6	-2.4	-0.9						
	AG	80	-5.5	-2.3	-0.1						
	AG	40	-5.7	-2.1	-1.6						
DAY 27	VIRUS	80	18.7	-1.1	0.9						
	VIRUS	40	17.2	-1.8	-0.8						
	AG	80	4.7	3.3	1.9						
	AG	40	1.3	1	1.1						
MIN/MAX			24.4	11.5	10.5						
CONTROL											
JC EA4	CD4	10	2.7	1.6	-1.3						
MIN/MAX			22.4	12.6	13.3						

ARMY #	273						PROLIF. INDEX			PRNT	
UMMC #	58						VIRUS	AG		DAY 0	DAY 27
GROUP	1A	TSI-GSD INTRADERM WITH ALCOHOL WIPE					4.4	15.2		5	3
			PERCENT SPECIFIC IMMUNE LYSIS								
			OF ^{51}Cr AUTOLOGOUS BLCL								
		E/T	VAC-VIR	VAC-AG	UNINF						
DAY 0	VIRUS	80	-1.6	-1.2	-1.3						
	VIRUS	40	-1.8	-1.6	-0.9						
	AG	80	-2.4	-0.8	-1.5						
	AG	40	-0.9	-1.1	-2						
DAY 27	VIRUS	80	27.5	-2.3	-1.4						
	VIRUS	40	29.1	-2.5	-2.7						
	AG	80	-1.4	-3.3	-2.8						
	AG	40	-4.8	-3.5	-3.2						
MIN/MAX			23.7	13	15						
CONTROL											
JC EA4	CD4	10	51.2	33.3	-8						
MIN/MAX			24.2	18	19						

ARMY #	282						PROLIF. INDEX			PRNT		
UMMC #	60						VIRUS	AG		DAY 0	DAY 27	
GROUP	2	TSI-GSD INTRAMUSCULARLY					13.9	29.1		12	3	
			PERCENT SPECIFIC IMMUNE LYSIS									
			OF ^{51}Cr AUTOLOGOUS BLCL									
		E/T	VAC-VIR	VAC-AG	UNINF		DAY 27		E/T	VAC-VIR	UNINF	K562
							VIRUS BULK					
DAY 0	VIRUS	80	16.2	1.2	0.1		NO C'		80	62.8	4.9	36.3
	VIRUS	40	7.4	-0.1	-0.1		C' ONLY		80	65.2	4.1	26.6
	AG	80	15.8	0.1	0.9		C' + ANTI-CD3		80	15.4	0.7	17
	AG	40	10.6	-0.3	0.8		C' + ANTI-CD4		80	55.6	1.3	23.3
							C' + ANTI-CD8		80	2.9	-2.1	14
DAY 27	VIRUS	80	77.6	5.8	6.7		C' + ANTI-CD16		80	59.7	-1.3	2.1
	VIRUS	40	69	3.5	4							
	AG	80	45.7	7.5	10.4		ANTIGEN BULK					
	AG	40	29.9	6.8	4.7		NO C'		80	NT	NT	NT
							C' ONLY		80	18.6	4.6	53.7
MIN/MAX			17.7	8.5	10.4		C' + ANTI-CD3		80	15.6	1.5	51.8
							C' + ANTI-CD4		80	16.7	3.1	40.7
							C' + ANTI-CD8		80	12.2	1.6	49.5
							C' + ANTI-CD16		80	8.9	4.2	9.3
CONTROL												
JC DC7	CD8	10	34.7	0.3	0.1		MIN/MAX			18.6	13.2	5.1
JC EA4	CD4	10	50.8	24.5	-0.6							
MIN/MAX			30.7	8.5	9.4							

ARMY #	292						PROLIF. INDEX			PRNT		
UMMC #	44						VIRUS	AG		DAY 0	DAY 27	
GROUP	1B	TSI-GSD INTRADERM W/OUT ALCOHOL WIPE					27.6	48.3		5	277	
			PERCENT SPECIFIC IMMUNE LYSIS									
			OF ^{51}Cr AUTOLOGOUS BLCL									
		E/T	VAC-VIR	VAC-AG	UNINF		DAY 27		E/T	VAC-VIR	UNINF	K562
							VIRUS BULK					
DAY 0	VIRUS	80	-5.4	-1.3	-0.5		NO C'		80	64.5	7.6	49.3
	VIRUS	40	-4.3	-2.5	-2.1		C' ONLY		80	62.8	5.8	43.5
	AG	80	-3	-2.4	-1.9		C' + ANTI-CD3		80	24.4	0.8	38
	AG	40	-3.4	-2.7	-1		C' + ANTI-CD4		80	52.1	1.6	38.8
							C' + ANTI-CD8		80	1.6	2.2	35
DAY 27	VIRUS	80	77.1	12.9	6		C' + ANTI-CD16		80	61.8	5.2	19.5
	VIRUS	40	55.9	9.5	4.2							
	AG	80	52.1	10.9	4.1		ANTIGEN BULK					
	AG	40	35.9	11.6	3.2		NO C'		80	22.7	7	64.3
							C' ONLY		80	24	8	65.5
MIN/MAX			17.2	12	12.5		C' + ANTI-CD3		80	-0.5	1	42.2
							C' + ANTI-CD4		80	-0.5	0.2	52.8
							C' + ANTI-CD8		80	8.7	3.6	45.7
							C' + ANTI-CD16		80	7.8	4.4	28.2
CONTROL												
JC EA4	CD4	10	63.7	31.3	-2.7		MIN/MAX			23.3	18	6.8
MIN/MAX			18.4	12	12.8							

ARMY #	288						PROLIF. INDEX			PRNT	
UMMC #	53						VIRUS	AG		DAY 0	DAY 27
GROUP	2	TSI-GSD INTRAMUSCULARLY					7.4	5.2		3	65
			PERCENT SPECIFIC IMMUNE LYSIS								
			OF ^{51}Cr AUTOLOGOUS BLCL								
		E/T	VAC-VIR	VAC-AG	UNINF						
DAY 0	VIRUS	80	5.9	0.9	0.3						
	VIRUS	40	-0.6	-0.9	0						
	AG	80	7	2.3	1						
	AG	40	7.1	0.8	-0.4						
DAY 27	VIRUS	80	77.4	16.8	6.4						
	VIRUS	40	78.1	12	4.3						
	AG	80	28.4	19.8	8.2						
	AG	40	27.6	14.3	2.8						
MIN/MAX			17.7	10.7	11.5						
CONTROL											
JC EA4	CD4	10	51.2	33.3	-8						
MIN/MAX			24.2	18	19						

ARMY #	294						PROLIF. INDEX			PRNT		
UMMC #	73						VIRUS	AG		DAY 0	DAY 27	
GROUP	3	WYETH					23.9	36.2		5	664	
			PERCENT SPECIFIC IMMUNE LYSIS									
			OF ^{51}Cr AUTOLOGOUS BLCL									
		E/T	VAC-VIR	VAC-AG	UNINF		DAY 27		E/T	VAC-VIR	UNINF	K562
							VIRUS BULK					
DAY 0	VIRUS	80	-4.5	-2.4	-0.8		NO C'		80	NT	NT	NT
	VIRUS	40	-5.6	-1.3	-2.2		C' ONLY		80	31.8	-3.7	46.4
	AG	80	-5.4	-1.5	-0.2		C' + ANTI-CD3		80	-4.5	-4.6	26.9
	AG	40	-3.7	-2.1	-3.1		C' + ANTI-CD4		80	30.5	-4.5	40.4
							C' + ANTI-CD8		80	-2	-4.9	36.6
DAY 27	VIRUS	80	64.4	15.4	6.6		C' + ANTI-CD16		80	32.6	-4.7	-2.2
	VIRUS	40	58.3	11.4	6.7							
	AG	80	22.5	13.7	4.8		ANTIGEN BULK					
	AG	40	10.5	11.6	4.1		NO C'		80	NT	NT	NT
							C' ONLY		80	1.3	-2.5	17.7
MIN/MAX			19.7	12	13.6		C' + ANTI-CD3		80	-3.8	-3.9	17
							C' + ANTI-CD4		80	-6.1	-4.3	9.8
							C' + ANTI-CD8		80	-0.9	-3.8	13.2
							C' + ANTI-CD16		80	0.9	-3	-0.3
CONTROL												
JC EA4	CD4	10	2.7	1.6	-1.3		MIN/MAX			19.2	15.5	12
MIN/MAX			22.4	12.6	13.3							

ARMY #	297						PROLIF. INDEX			PRNT		
UMMC #	37						VIRUS	AG		DAY 0	DAY 27	
GROUP	3	WYETH					47.8	34.5		5	615	
			PERCENT SPECIFIC IMMUNE LYSIS									
			OF ^{51}Cr AUTOLOGOUS BLCL									
		E/T	VAC-VIR	VAC-AG	UNINF		DAY 27		E/T	VAC-VIR	UNINF	K562
							VIRUS BULK					
DAY 0	VIRUS	80	5.1	-1.1	-0.4		NO C'		80	NT	NT	NT
	VIRUS	40	7.2	-0.6	-0.9		C' ONLY		80	61.2	-0.2	45.3
	AG	80	4.1	-0.7	0.4		C' + ANTI-CD3		80	46.9	1.8	52.2
	AG	40	5	-1.5	-0.5		C' + ANTI-CD4		80	62	0.2	37
							C' + ANTI-CD8		80	9.1	0.1	42.3
DAY 27	VIRUS	80	83.3	21.5	9		C' + ANTI-CD16		80	49.6	0	6.8
	VIRUS	40	75.4	16.7	6							
	AG	80	58.1	20.7	4.2		ANTIGEN BULK					
	AG	40	33.9	16.7	2.2		NO C'		80	NT	NT	NT
							C' ONLY		80	14.9	-0.4	33.4
MIN/MAX			13.1	9.1	9.2		C' + ANTI-CD3		80	2.2	-1.7	36
							C' + ANTI-CD4		80	-0.1	0.9	19.7
							C' + ANTI-CD8		80	14.6	0.7	27.4
							C' + ANTI-CD16		80	23.4	1	6.8
CONTROL												
JC DC7	CD8	10	35.9	-1.9	-1.4		MIN/MAX			16.6	12.9	8.6
JC EA4	CD4	10	37.8	35.7	-1							
MIN/MAX			15.3	12.9	12.3							

ARMY #	302						PROLIF. INDEX			PRNT		
UMMC #	41						VIRUS	AG		DAY 0	DAY 27	
GROUP	3	WYETH					17.5	4		3	346	
			PERCENT SPECIFIC IMMUNE LYSIS									
			OF ^{51}Cr AUTOLOGOUS BLCL									
		E/T	VAC-VIR	VAC-AG	UNINF		DAY 27		E/T	VAC-VIR	UNINF	K562
							VIRUS BULK					
DAY 0	VIRUS	80	-6.6	-4.4	-3.7		NO C'		45	NT	NT	NT
	VIRUS	40	-7.6	-3.2	-3		C' ONLY		45	28.9	3.1	58
	AG	80	-2.8	-4	-2.9		C' + ANTI-CD3		45	11.4	2.4	39.7
	AG	40	4.7	-2.9	-3		C' + ANTI-CD4		45	25	3	42
							C' + ANTI-CD8		45	10.5	2.7	48
DAY 27	VIRUS	80	52.4	4.1	-1.6		C' + ANTI-CD16		45	29.3	6	60.3
	VIRUS	40	57.2	3.1	0							
	AG	80	54.9	7	0.1		ANTIGEN BULK					
	AG	40	37.2	4.3	-1.1		NO C'		80	19.8	2.6	48
							C' ONLY		80	19.9	2.7	49.2
MIN/MAX			23.2	14.9	13.1		C' + ANTI-CD3		80	0.1	2.7	17.1
							C' + ANTI-CD4		80	-1.5	2.4	35.3
							C' + ANTI-CD8		80	16.1	2.9	49.2
							C' + ANTI-CD16		80	18.8	2.9	5.4
CONTROL												
JC EA4	CD4	10	63	31.3	-2.7		MIN/MAX			23.3	10.7	6.8
MIN/MAX			18.4	12	12.8							

ARMY #	303						PROLIF. INDEX			PRNT		
UMMC #	38						VIRUS	AG		DAY 0	DAY 27	
GROUP	3	WYETH					16.1	23.9		7	206	
			PERCENT SPECIFIC IMMUNE LYSIS									
			OF ^{51}Cr AUTOLOGOUS BLCL									
		E/T	VAC-VIR	VAC-AG	UNINF		DAY 27		E/T	VAC-VIR	UNINF	K562
							VIRUS BULK					
DAY 0	VIRUS	80	1.1	-1	-1.8		NO C'		80	32.5	-1	21.4
	VIRUS	40	0.5	-2.6	-2.8		C' ONLY		80	32.7	-1.5	23.3
	AG	80	1.6	-1.7	0.1		C' + ANTI-CD3		80	8.7	-3	19.5
	AG	40	-0.2	-2.4	-2.1		C' + ANTI-CD4		80	36.2	2.5	27.6
							C' + ANTI-CD8		80	6.1	-3.4	18.6
DAY 27	VIRUS	80	72.3	29.5	7.3		C' + ANTI-CD16		80	22.8	-0.5	3
	VIRUS	40	74	27.8	3.7							
	AG	80	55.6	24.1	3.1		ANTIGEN BULK					
	AG	40	33.3	21.7	0.3		NO C'		80	16	-2.7	13
							C' ONLY		80	8.2	-2.7	6.8
MIN/MAX			16.4	12.5	13.7		C' + ANTI-CD3		80	-0.2	-1.9	4.3
							C' + ANTI-CD4		80	0.2	-1	6.1
							C' + ANTI-CD8		80	4.7	-3.8	5.4
							C' + ANTI-CD16		80	8.1	-3.2	1
CONTROL												
JC EA4	CD4	10	46.1	37.3	-1.2		MIN/MAX			28.4	20.2	6.6
MIN/MAX			16.6	10	10.8							

ARMY #	304						PROLIF. INDEX			PRNT		
UMMC #	40						VIRUS	AG		DAY 0	DAY 27	
GROUP	1B	TSI-GSD INTRADERM W/OUT ALCOHOL WIPE					18.4	64		3	167	
			PERCENT SPECIFIC IMMUNE LYSIS									
			OF ^{51}Cr AUTOLOGOUS BLCL									
		E/T	VAC-VIR	VAC-AG	UNINF		DAY 27		E/T	VAC-VIR	UNINF	K562
							VIRUS BULK					
DAY 0	VIRUS	80	-0.5	-0.4	-1.4		NO C'		80	23.2	-1.8	17.8
	VIRUS	40	0.7	-1.7	-1.9		C' ONLY		80	30.7	2.3	21.1
	AG	80	-0.9	-1.4	-1.3		C' + ANTI-CD3		80	-1.8	-5.8	8.6
	AG	40	2.8	-0.9	-1.7		C' + ANTI-CD4		80	35.8	-0.8	15
							C' + ANTI-CD8		80	-0.9	-5.7	11.3
DAY 27	VIRUS	80	50.7	7.3	2		C' + ANTI-CD16		80	38.5	-0.9	3.2
	VIRUS	40	48.9	3.9	0.5							
	AG	80	35.5	11	0.6		ANTIGEN BULK					
	AG	40	18.6	8.4	-0.5		NO C'		80	8.3	-2.5	20.4
							C' ONLY		80	7.7	-1.8	19.4
MIN/MAX			12	11.1	11.6		C' + ANTI-CD3		80	-3.5	-3.7	8.9
							C' + ANTI-CD4		80	-3	-5.5	8.5
							C' + ANTI-CD8		80	6.8	-7.5	15.8
							C' + ANTI-CD16		80	6.3	-2.7	4.4
CONTROL												
JC EA4	CD4	10	46.1	37.3	-1.2		MIN/MAX			20.7	23.7	6.6
MIN/MAX			16.6	10	10.8							

ARMY #	309						PROLIF. INDEX			PRNT	
UMMC #	48						VIRUS	AG		DAY 0	DAY 27
GROUP	1B	TSI-GSD INTRADERM W/OUT ALCOHOL WIPE					8	10.8		3	347
			PERCENT SPECIFIC IMMUNE LYSIS								
			OF ^{51}Cr AUTOLOGOUS BLCL								
		E/T	VAC-VIR	VAC-AG	UNINF						
DAY 0	VIRUS										
	VIRUS	80	-2.1	-3.7	-3						
	AG	40	-2.9	-4.2	-3.9						
	AG	80	0.5	-2.2	-3.1						
		40	2.1	-2.2	-2						
DAY 27	VIRUS										
	VIRUS	80	71.2	24	3.4						
	AG	40	81.1	18.7	2.2						
	AG	80	45.3	7	3.9						
		40	34.8	3.6	-0.9						
MIN/MAX											
			14.8	13.6	12.8						
CONTROL											
JC EA4	CD4	10	25.4	55.8	-3						
MIN/MAX			10.6	12.6	13.9						

ARMY #	326						PROLIF. INDEX			PRNT		
UMMC #	104						VIRUS	AG		DAY 0	DAY 27	
GROUP	3	WYETH					12.1	13.3		3	48	
			PERCENT SPECIFIC IMMUNE LYSIS									
			OF ^{51}Cr AUTOLOGOUS BLCL									
		E/T	VAC-VIR	VAC-AG	UNINF		DAY 27		E/T	VAC-VIR	UNINF	K562
							VIRUS BULK					
DAY 0	VIRUS	80	5.7	-0.8	-0.2		NO C'		80	NT	NT	NT
	VIRUS	40	NT	NT	NT		C' ONLY		80	NT	NT	NT
	AG	80	9.7	-0.4	-0.3		C' + ANTI-CD3		80	NT	NT	NT
	AG	40	NT	NT	NT		C' + ANTI-CD4		80	NT	NT	NT
							C' + ANTI-CD8		80	NT	NT	NT
DAY 27	VIRUS	80	59.3	3.7	2.3		C' + ANTI-CD16		80	NT	NT	NT
	VIRUS	40	63.1	5.5	6							
	AG	80	31.9	8.2	7.4		ANTIGEN BULK					
	AG	40	13.4	7.4	6		NO C'		80	NT	NT	NT
							C' ONLY		80	16.1	4.8	53.6
MIN/MAX			15.8	9.4	8.5		C' + ANTI-CD3		80	-0.9	10.6	48.3
							C' + ANTI-CD4		80	-3	8.7	41.6
							C' + ANTI-CD8		80	6.3	2.6	46.4
							C' + ANTI-CD16		80	5.4	1.4	25.5
CONTROL												
JC EA4	CD4	10	46.2	62.7	-2.3		MIN/MAX			12.6	12	10.2
MIN/MAX			16.1	14.7	15.8							

2. Measure antibodies in day 0 and day 28 sera from volunteers in the above study.

We have completed the immunoblot analyses on the day 0 and day 28 sera from all of the volunteers in the study. Generally, the immunoblot results correspond well to the neutralization titers obtained for the same sera by Dr. D. McClain. The data are shown in the last two columns in Tables 4-8. The data were generated by observation of the absence or presence of antibody reactivity to vaccinia antigen or western blots and were recorded as a - or a + in the Tables respectively.

The immunoblot procedure was described in detail in the original grant. Briefly, vaccinia virus infected CV-1 cell extracts are boiled for 5 min in sample buffer, loaded onto 12% SDS-polyacrylamide gels, and electrophoresed. After electrophoresis, the proteins are transferred to nitrocellulose using the semidry blotting apparatus from Gelman Sciences. The filters are then incubated with antisera (1:50 dilution) in BLOTTO overnight at 4°C with shaking, washed four times for 10 min each with phosphate-buffered saline (PBS), and further incubated with either goat anti-human (1:1,000) or goat anti-rabbit (1:500) antibody conjugated to horseradish peroxidase for 2 h at room temperature. After four more 10-min washes with PBS, the filters are developed with 0.2% 1-chloro-4-naphthol 0.006% hydrogen peroxide in PBS in order to visualize bound antibodies.

Initially there were immunoblots on 10 sera which were inconsistent with the PRNT data. The neutralization titers were low but the western blot results were positive (pre for Army subjects #274, 326, and 328; pre and D 27 for donor 270) or the neutralization titers were high but the western blot results were negative (D27 for Army subjects #20, 33, 280, 287, 288, and 319). Because of these inconsistences, these immunoblots were repeated. The repeat immunoblots were performed using slight modifications of the original protocol to enhance sensitivity and reduce background. Briefly, this vaccinia virus antigen was prepared using more purified virus by centrifugation of virion containing antigen at 14,000 g for 30 minutes. Pelleted virus was then boiled and applied to SDS-PAGE. After Western blotting, these samples were incubated with serum diluted 1:30 with 5% nonfat dry milk in PBS for 16-24 hours at 4°C. Previous results were generated using vaccinia antigen that were not centrifuged, and serum were tested at a dilution of 1:50.

Using this more sensitive approach, we found that the serum from donors (Army#'s 70, 288, 289 and 319 are now positive for reactivity on day 27. A repeat immunoblot analysis on the serum samples from donors 274, 326 and 328 indicate that these sera are negative on day 0 and positive on day 27. The sera from donor 270 appear to be negative on both days. The results obtained from serum samples from donors 33 and 280 were consistent with our previous results. We are confident with the results of this immunoblot analysis. During the initial analysis in some cases it was difficult to evaluate the immunoblot results due to background and/or low sensitivity.

Due to the inconsistency on Army donor #280 serum samples, we performed a plaque reduction neutralizing titer assay from day 0 and day 27 and determined that this donor had no plaque reduction at low serum dilution (1:5). The PRNT value reported to us by the Army for day 27 (198) appears to be inconsistent with our results.

3. Using high-responder PBMC, identify several proteins on vaccinia virus that are stimulating in bulk cultures.

Using high responder PBMC we have attempted to identify proteins encoded by vaccinia virus that stimulate human CD4+ and CD8+ CTL in bulk culture. These studies were performed on a part-time basis as an extension of time.

A. One strategy we began to develop was to generate HLA-A2 restricted CD8+ CTL clones from HLA-A2 haplotype bearing vaccinia-immune donors to use in CTL assays against target cells pulsed with HPLC purified peptide fractions eluted from MHC HLA-A2 columns. Vaccinia virus-infected cell extracts are first put over the column. Bound peptides, presumably those that have the HLA-A2 binding motif, are eluted and HPLC purified. We planned to test these peptides fractions with our CD8+ HLA-A2 restricted CTL clones. Dr. Ken Parker, a postdoctoral fellow in Dr. J. Colligan's lab at the NIH, was to provide us with the HPLC purified HLA-A2 vaccinia peptide fractions.

As an example, one of our vaccinia-immune donors (VA16) has the HLA-A2 allele. This donor was immunized against smallpox with vaccinia virus approximately 55 years ago. PBMC from this donor were initially stimulated with anti-CD3 and allogeneic feeders. Fourteen and 21 days later the culture was stimulated with live vaccinia virus and autologous irradiated PBMC. Results of a CTL assay performed 7 days after the last stimulation with virus appear in Table 9.

TABLE 9: TARGET CELL LYSIS (%)

Effectors (E:T)	VA16 B-LCL	VA16 B-LCL + VAC	K562
VA 16 Bulk Culture (50:1)	3.0	43.3	3.3
VA 16 Bulk Culture (25:1)	4.3	44.7	3.8

The results from this bulk culture CTL assay demonstrate convincing vaccinia virus-specific CTL activity. The cells were stained with a FITC conjugated monoclonal antibody to CD8+ and sorted by FACS. The sorted CD8+ T cells were then limit diluted at several cell concentrations in 96 well plates. The microcultures were stimulated every two weeks with anti-CD3 and irradiated allogeneic feeder cells. Several clones were identified after initial screening against vaccinia virus-infected autologous B-LCL and 5 clones have maintained significant levels of vaccinia virus-specific CTL activity after secondary screening (Table 10).

TABLE : 10

Effectors (E:T=25:1)	TARGET CELL LYSIS (%) VA16 B-LCL	VA16 B-LCL + VAC
B33	-1.5	49.9
B34	-2.5	48.4
B35	-1.6	36.9
C7	0.8	33.7
C8	9.2	22.3

Two of the clones were tested in a CTL assay utilizing vaccinia virus-deletion mutants. These mutant viruses contain various size deletions. The rationale is: if a particular clone is unable to lyse autologous targets infected with a deletion mutant, then the epitope recognized by the clone is contained within the deleted region.

The deletion mutants are described in Table 11.

TABLE 11:

VAbT 213: deletion of 420 base pairs within the thymidine kinase gene
VAbT 247: deletion of 20,000 base pairs at the 5' end of the virus genome
VP 866: deletion of 18 open reading frames as described by Tartaglia et al, (1992) Virology 188:217-232.
VP 811: deletion of 55 open reading frames as described by Perkus et al, (1991) Virology 180:406-410.

Clones B33 and B34 recognized and lyse each of the targets infected with the deletion mutants therefore the epitopes recognized by these CTL's were not contained within the deleted regions (Table 12). Additional experiments utilizing the clones B33, C7 and C8 are to be performed. We will attempt identification of the HLA restricting allele for each of the clones by using HLA partially matched allogenic B-LCL infected with vaccinia virus as targets in CTL assays.

TABLE 12:

Effectors (E:T 25:1)	TARGET CELL LYSIS (%) BLCL	BLCL+VAC	BLCL+VAbT213	BLCL+VABT247	BLCL+VP866	BLCL+VP811
B33	-2.2	30.9	32.1	42.4	23.5	26.7
B34	-2.9	24.3	26.9	34.5	28.3	28.3

B. Another approach that we have utilized involves the generation of vaccinia virus-specific CTL clones from the PBMC of selected donors in the Army Vaccine Study which we have

reported previously. We have chosen 4 donors that had significant levels of CTL lysis of autologous B-LCL infected with vaccinia virus. PBMC from donors (Army #'s 34, 262, 282 and 292) were stimulated with live virus and used in a CTL assay on day 7 (Table 13).

TABLE 13:

EFFECTORS		TARGET CELL LYSIS (%)	
Donor	E:T	Autologous B-LCL	Autologous B-LCL + Vac
Army #34	80:1	1.7	65.8
	40:1	0.9	51.3
Army #262	80:1	5.1	13.5
	40:1	1.8	8.1
Army #282	80:1	1.3	49.2
	40:1	1.1	37.9
Army #292	80:1	4.9	8.2
	40:1	2.3	4.0

Donors 34 and 282 had significant levels of vaccinia virus-specific CTL activity. We performed limiting dilutions on the vaccinia specific bulk cultures generated from donors 34 and 282 at various cell concentrations per well. After 5 weeks the plates were discarded due to contamination, and the study reached its termination date.

CONCLUSIONS

This Phase II clinical trial was initiated by the Army to study the humoral and cellular immune responses generated from immunization with live experimental and standard smallpox vaccines given by different routes.

The bulk culture lymphocyte proliferation data obtained during the first year of this study indicate that the experimental in vitro tissue-culture derived vaccine that scientists at USAMRIID developed is immunogenic in most recipients; however it is less immunogenic than the standard smallpox vaccine. The results (summarized in table 3) indicate that individuals in all of the vaccine groups had vaccinia-specific T-lymphocyte responses. The three vaccine groups that received the TSI-GSD241 vaccine had similar vaccinia specific immune responses, whether the vaccine was given intradermally, with or without an alcohol wipe, or intramuscularly. The standard Wyeth vaccine given by scarification induced more vigorous T-lymphocyte responses in virtually all of the volunteers in that group.

The plaque reduction neutralizing antibody titer values shown in Tables (4-8) were provided to us by Dr. D. McClain of USAMRIID. Our immunoblot results correlate very well

with the PRNT data. Only immunoblot data from one donor, Army #83, was inconsistent with the PRNT data. Overall our immunoblot data and the PRNT data correlated well with the vaccinia virus specific T cell memory responses detected in this study.

Upon completion of the analysis of vaccinia virus-specific cytotoxic T cell memory activity in the PBMC from donors in this study, it became clear that both vaccines stimulated vaccinia virus-specific CTL response in vivo. Bulk cultures derived from donors who received the standard Wyeth Dryvax vaccine given by cutaneous inoculation had significantly higher levels of CTL lytic activity than those cultures derived from donors who received the TSI-GSD24 vaccine regardless of the administration route. Complement depletion analysis on selected cultures demonstrated that both CD4+ and CD8+ CTL responses were generated by the vaccines in vivo because in vitro stimulation of donor PBMC with live virus preferentially expanded vaccinia virus-specific CD8+ CTL and stimulation with inactivated vaccinia virus antigen preferentially gave rise to vaccinia virus specific CD4+ CTL. The experiments performed in option year 1 to establish approaches for the identification of T cell epitopes on vaccinia virus encoded proteins began to generate interesting data; however, the project reached its termination date.

Generally, the standard Wyeth Dryvax smallpox vaccine stimulated more vigorous humoral and cellular immune responses than the experimental TSI-GSD241 vaccine. The reasons for the less than optimal stimulation of the vaccinia virus-specific immune response by the experimental tissue culture derived vaccine are not known, however, the ID and IM routes of administration are very different from the cutaneous route of inoculation utilized when administering the standard Wyeth Dryvax vaccine and these differences are likely to be significant. Clearly the route of administration would influence which cell types actually presented vaccinia antigens to the immune system and directly influence it immunogenicity. Additionally vaccinia virus replicates at different rates depending on the cell type and therefore the route of inoculation would influence the vaccines ability to replicate. Good replication of the vaccine virus as demonstrated by the appearance of vesicles at the vaccine site seems to correlate with the magnitude of the immune response. No vaccinees that received the experimental vaccine through the IM route and only a few that received the vaccine ID developed vesicles at the site of inoculation (15). Those that did generated vaccinia-specific immune responses that were comparable to those generated by scarification. In summary, the IM route of immunization stimulated the least efficient immune response. The ID route, regardless of an alcohol wipe, generated an intermediate response (with a better response correlated with the development of a vesicle at the vaccine site) and an apparent optimal response was stimulated through scarification with the standard smallpox vaccine.

Another obvious difference between the two vaccines is their origin. TSI-GSD241 was generated by propagation in vitro whereas the standard Wyeth Dryvax smallpox vaccine was produced in vivo and isolated from vesicles at the inoculation site on calves. Propagation of the TSI-GSD241 vaccine in vitro may have caused it to become attenuated and as a result, less immunogenic.

A direct comparison of the same routes of administration between the standard Wyeth vaccine and the TSI-GSD241 vaccine was not done, i.e. the desire to develop a vaccine that

would be given by injection to avoid safety and transmission concerns which have occurred after administering standard vaccine by the recommended scarification route. It is very possible that the more modest immune responses induced by the TSI-GSD241 vaccine are due to the routes of immunization and it may have stimulated a more vigorous immune response had it been administered by scarification. It is also possible that increasing the experimental vaccine dose or performing multiple inoculations may increase its potency, however, either of these approaches would create additional issues and are not likely to be desirable. Immunization through cutaneous inoculation may be less attractive from a safety standpoint, however, all the data generated as a result of this study suggests that route of immunization is the only route capable of stimulating consistent optimal vaccinia virus-specific humoral and cellular immune responses.

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List of personnel that received pay from the negotiated effort:

Verla Berrios

Margaret Berryman

Walter Demkowicz

Francis Ennis

Susan Gagnon

Anita Leporati

Anuja Matthew

Leslie Wu

Di Zhu

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APPENDIX

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Human Cytotoxic T-Cell Memory: Long-Lived Responses to Vaccinia Virus

WALTER E. DEMKOWICZ, JR.,* REBECCA A. LITTAUA, JIANMING WANG, AND FRANCIS A. ENNIS

Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts 01655

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Peripheral T lymphocytes can be classified into two groups: naive and memory T cells. The focus of this study was to examine the duration of T-cell memory in humans. Vaccinia virus replicates in the cytoplasm of infected cells and is not thought to persist or become latent after the acute phase of infection. We identified long-lived vaccinia virus-specific memory cytotoxic T cells in adults who had been immunized against smallpox as children. Initially, we detected vaccinia virus-specific T cells in peripheral blood mononuclear cells while screening for human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses in HIV-1-seropositive subjects. These individuals had not had contact with vaccinia virus since their primary immunization in early childhood. Several vaccinia virus-specific $CD4^+$ T-cell clones were derived from these donors and characterized. Healthy, HIV-1-seronegative donors who had been immunized against smallpox many (35 to 50) years earlier were also screened for vaccinia virus-specific T-cell immunity. We found significant $CD8^+$ and $CD4^+$ cytotoxic T-cell responses to vaccinia virus after in vitro stimulation, indicating that these memory cells are maintained in vivo for many years. The peripheral blood mononuclear cells of young adults with no history of immunization against smallpox did not develop vaccinia virus-specific T-cell responses after in vitro stimulation. Precursor frequency analysis of the vaccinia virus-specific memory $CD4^+$ T cells from a donor immunized with vaccinia virus 35 years earlier revealed a frequency of 1 in 65,920 $CD4^+$ T cells. We concluded that specific vaccinia virus T-cell immunity can persist for up to 50 years after immunization against smallpox in childhood in the presumed absence of exposure to vaccinia virus.

The ability of a T cell to recognize a specific peptide epitope in the context of a major histocompatibility complex molecule on the surface of virus-infected cells is provided by its T-cell receptor (15, 16). The interaction between the T-cell receptor and the antigen-major histocompatibility complex triggers proliferation and clonal expansion of specific T cells (15, 16). The proliferative T-cell response continues during viral infections until the cells expressing the viral epitopes are eliminated or the virus becomes latent within cells and is no longer detectable by T cells. Some of the progeny of the antigen-responsive T cells develop into antigen-specific memory T cells. This subpopulation of T cells is maintained within the host and provides immune surveillance. In the event of reactivation of latent virus and expression of viral antigens, or subsequent natural reexposure to the virus, specific memory T cells become activated and clonally expand with greater magnitude than during the initial response. It is generally accepted that specific immune T-cell memory persists after an encounter with an antigen and may help to protect the immune host against subsequent exposure to that pathogen. The underlying mechanisms which contribute to immunological T-cell memory are poorly understood and have only recently received much attention (11, 14, 17, 20, 22).

Vigorous and long-lasting protective immune responses have long been associated with smallpox vaccination. After a single immunization with vaccinia virus, virus-specific $CD4^+$ and $CD8^+$ cytotoxic T lymphocytes (CTL) are generated, as evidenced by in vitro restimulation of memory T cells (7, 19). Specific immunity is believed to be maintained for many years, and booster immunizations are recommended every 10 years for those at risk for infection. Because of the decreased risk of smallpox infection and side effects of vaccination, vaccinia virus has not been generally used since routine vaccinations were discontinued over 20 years ago. Laboratory workers using vaccinia virus and some members of the military continue to be vaccinated. Vaccinia virus does not cause persistent or latent infections, and therefore repeated endogenous antigenic stimulation is not thought to occur (5). These characteristics of vaccination against smallpox led us to investigate the longevity of vaccinia virus-specific memory responses in humans.

Peripheral blood mononuclear cells (PBMC) of human immunodeficiency virus type 1 (HIV-1)-seropositive donors exhibit vaccinia virus-specific cytotoxic activity against autologous B-LCL cells expressing vaccinia antigen. Cryopreserved PBMC from 22 asymptomatic HIV-1-seropositive donors and 8 HIV-1-seronegative, healthy donors were tested directly in a ^{51}Cr release assay as previously described for HIV-1 envelope antigen-specific cytotoxicity by using autologous B-LCL target cells that were either uninfected or infected with vaccinia virus or the recombinant V/gp160 (4, 18). We detected significant HIV-1 envelope antigen-specific cytotoxicity in the PBMC of 2 (9%) of the HIV-1-seropositive donors tested (data not shown), but significant lysis of target cells expressing vaccinia virus antigens was observed using the PBMC of 6 (27%) of the HIV-1-seropositive donors, as shown in Table 1. K562 cells were used as target cells to measure natural killer cell activity.

Establishment of vaccinia virus-specific $CD4^+$ CTL lines. Several cell lines were obtained that specifically lysed vaccinia virus-infected target cells after stimulation of isolated PBMC with γ-irradiated allogeneic PBMC, anti-CD3 monoclonal antibody 12F6 (0.1 μg/ml), kindly supplied by J. Wong as previ-

* Corresponding author. Mailing address: Division of Infectious Diseases & Immunology, Department of Medicine, University of Massachusetts Medical Center, 55 Lake Ave. North, Worcester, MA 01655. Phone: (508) 856-6303. Fax: (508) 856-4890.

TABLE 1. Vaccinia virus-specific cytotoxic activity in PBMC of asymptomatic HIV-1-seropositive donors[a]

Donor no.	% Specific lysis of target cells			
	Uninfected B-LCL cells	Vaccinia virus-infected B-LCL cells	V/gp160-infected B-LCL cells	Uninfected K562 cells
2	17.2	29.8	23.8	1.8
11	8.9	31.8	29.0	1.7
12	10.1	25.9	18.4	2.3
13	2.8	13.5	15.4	2.1
26	7.0	17.4	13.1	2.5
27	2.3	17.8	15.4	3.3

[a] Unstimulated PBMC were tested directly in a CTL assay. Cytotoxicity was determined in a 6-h ^{51}Cr release assay effector-target cell at an ratio of 50:1. ^{51}Cr release was calculated by the formula 100 × (mean experimental release − mean spontaneous release)/(mean total release − mean spontaneous release). The results of an assay were excluded if the mean level of spontaneous release was >30%.

ously described ([28]), and recombinant human interleukin-2. These cell lines were expanded and subcloned by limiting dilution at 0.3, 1, or 3 cells per well. We isolated vaccinia virus-specific cytotoxic T-cell clones from each of three HIV-1-seropositive donors (Table 2). These cell lines lysed autologous target cells infected with vaccinia virus, and the lysis of K562 cells was low. Phenotypic analysis of these CTL lines revealed that they were all $CD3^+$ $CD4^+$ $CD8^-$ $Leu11^-$.

Demonstration of long-lived vaccinia virus-specific $CD8^+$ CTL responses. Presumably, the HIV-1-seropositive donors from whom the $CD4^+$ clones were generated had not been exposed to vaccinia virus since childhood vaccination, because the donors were over 30 years of age, had not served in the military, and had not been vaccinated since early in childhood. To determine whether vaccinia virus-specific CTL precursors are maintained in the peripheral blood of individuals for long periods of time after immunization in early childhood, two healthy HIV-1 antibody-negative donors, VA15 and VA16, were identified who were known to have been immunized as children with vaccinia virus 35 and 50 years earlier, respectively. These donors stated that they had no subsequent exposure to vaccinia virus or to any other poxvirus. We stimulated these two donors' PBMC with live vaccinia virus in vitro in an attempt to detect vaccinia virus-specific memory $CD8^+$ CTL because we had only isolated $CD4^+$ major histocompatibility complex class II-restricted CTL from the HIV-1-seropositive donors by using anti-CD3 antibody and interleukin-2 stimulations. This method of stimulation with live vaccinia virus had been used earlier to successfully generate vaccinia virus-specific $CD8^+$ major histocompatibility complex class I-restricted CTL in vitro (7).

Donor PBMC were stimulated in vitro with live vaccinia virus, which induced a modest level of vaccinia virus-specific proliferation, and the cultures were restimulated with anti-CD3 antibody on day 7. On day 14, the cultures were restimulated with live vaccinia virus and assayed on day 21 for cytotoxic activity. The bulk cultured cells derived from donors VA15 and VA16 lysed vaccinia virus-infected autologous B-LCL cells at levels of 50.8 and 44.2%, respectively, at an effector-target cell ratio of 50:1 (Table 3). Incubation of effector cells with anti-$CD3^+$ or anti-$CD8^+$ specific monoclonal antibodies in the presence of complement reduced the levels of lysis significantly, indicating that $CD8^+$ CTL were the major effector cells in this virus-stimulated population. There was some decrease in the level of lysis after treatment with either anti-$CD4^+$ antibodies and complement or anti-CD16 antibodies and complement, suggesting a contribution by vaccinia virus-specific $CD4^+$ CTL and NK cells (Table 3).

To confirm that the vaccinia virus-specific CTL activity observed in these cultures was not a result of primary in vitro stimulation, the PBMC of two healthy, young adult donors who had no history of immunization with vaccinia virus were used as controls. The PBMC from these donors, VA21 and VA23, were stimulated in a fashion identical to that used for the PBMC of donors VA15 and VA16, were assayed on day 21 for vaccinia virus-specific CTL activity, and had none (Table 3).

Frequency analysis of vaccinia virus-specific CTL memory cells. As described above, the PBMC isolated from donor VA15, who had been immunized with vaccinia virus more than 35 years ago, exhibited vaccinia virus-specific CTL activity after stimulation in vitro. We wanted to determine the frequency of vaccinia virus-specific memory T cells within the PBMC of this donor. We assumed that the precursor frequency of vaccinia virus-specific CTL would be low, so preliminary experiments were performed to ensure that we could detect a positive response. Sorting of donor PBMC by fluorescence-activated cell sorter analysis yielded a relatively pure population of $CD4^+$ T cells to assay for vaccinia virus-specific memory T cells. Microcultures were initiated under limiting-dilution conditions with 5,000 to 60,000 sorted $CD4^+$ T cells per well in 24 replicate wells. Each microculture received 2×10^5 γ-irradiated autologous PBMC in 200 μl of RPMI 1640 medium supplemented with 20% fetal calf serum, 40 U of interleukin-2 per ml, and vaccinia virus antigen. Vaccinia virus antigen was prepared by infecting a confluent monolayer of CV-1 cells with virus at a multiplicity of infection of 10. After 24 h of incubation, when the cytopathic effect was extensive, the cells were harvested by scraping. After a freeze-thaw cycle, sonication was performed and the cell-virus extract was boiled for 10 min to inactivate any residual infectious virus before use. Generally, a confluent monolayer of vaccinia virus-infected CV-1 cells in a 75-cm^2 tissue culture flask (approximately 12×10^6 cells) would yield 2 ml of cell-virus extract. At 3-day intervals, one-half of the culture medium was removed and replaced with

TABLE 2. Lysis of vaccinia virus-infected target cells by T-cell clones from HIV-1-seropositive donors[a]

Donor no.	Clone no.	% Specific lysis of target cells					
		Uninfected B-LCL cells	Vaccinia virus-infected B-LCL cells	V/gag-infected B-LCL cells	V/pol-infected B-LCL cells	V/gp160-infected B-LCL cells	Uninfected K562 cells
11	214	0	71.8	62.8	64.8	73.5	10.4
2	140	3.1	27.7	29.6	29.3	12.6	9.8
12	109	11.4	32.8	26.5	NT[b]	18.5	3.3

[a] Cytotoxicity was determined in a 5-h ^{51}Cr release assay at an effector-target cell ratio of 4:1. Percent specific ^{51}Cr release was calculated as described in Table 1, footnote *a*.

[b] NT, not tested.

TABLE 3. Characterization of bulk culture cytotoxicity activity

Donor (day) and treatment	% specific ^{51}Cr release from target cells[a]		
	Uninfected B-LCL cells	Vaccinia virus-infected B-LCL cells	Uninfected K562 cells
VA15 (21)			
Complement	−0.2	50.8	23.1
Anti-$CD4^+$ antibodies, complement	−0.3	32.9	14.7
Anti-$CD8^+$ antibodies, complement	−3.3	−5.5	9.8
Anti-$CD3^+$ antibodies, complement	−3.4	−5.1	2.7
Anti-$CD16^+$ antibodies, complement	3.8	37.4	13.0
VA16 (21)			
Complement	3.3	44.2	35.3
Anti-$CD4^+$ antibodies, complement	0.0	36.9	27.3
Anti-$CD8^+$ antibodies, complement	1.7	3.4	19.0
Anti-$CD3^+$ antibodies, complement	−1.3	−0.2	15.7
Anti-$CD16^+$ antibodies, complement	2.3	36.2	25.9
VA21 (21)			
Complement	−1.9	−3.5	0.3
Anti-$CD3^+$ antibodies, complement	−1.1	−4.9	1.1
Anti-$CD16^+$ antibodies, complement	−2.8	−2.7	0.4
VA23 (21)			
Complement	3.2	7.2	−0.1
Anti-$CD3^+$ antibodies, complement	0.6	3.3	−0.7
Anti-$CD16^+$ antibodies, complement	3.6	2.3	−0.2

[a] Effector-target cell ratio, 50:1.

fresh medium without vaccinia virus antigen as described above. On day 10, each well was split and cells were assayed for cytotoxicity on autologous uninfected B-LCL cells or B-LCL cells infected with vaccinia virus. Individual wells were considered positive if the calculated specific lysis of the virus-infected target cells was greater than 3 standard deviations above the mean levels of lysis calculated from negative wells. Precursor frequencies were calculated by using χ^2 analysis as described by Taswell (27), by using a computer program kindly provided by Richard Miller (University of Michigan, Ann Arbor). Analysis of the precursor frequency of $CD4^+$ vaccinia virus-specific memory T cells resulted in a calculated frequency of 1 in 65,920 sorted $CD4^+$ T cells (95% confidence interval, 48,731 to 101,844 cells) or 3 in 10^6 PBMC (Fig. 1). Three experiments were performed on the sorted $CD4^+$ T cells, and the calculated frequencies for each experiment were consistent, differing by less than 2%. This calculated frequency is lower than the reported precursor frequencies for varicella-zoster virus-, HIV-1-, and cytomegalovirus-specific T cells (3, 12, 13), but those viruses cause persistent and/or latent infections, unlike vaccinia virus.

The long-lived T-cell memory responses we observed are striking; however, a recent study suggested that memory T-cell responses to vaccinia virus may be long lasting. In a randomized phase 1 trial reported by Cooney et al., 35 healthy, HIV-1-seronegative, young adult males, 31 of whom had a history of smallpox immunization and 4 of whom were vaccinia virus naive, were immunized with a recombinant vaccinia virus vaccine expressing the gp160 envelope gene of HIV-1 (6). Individuals who had been immunized as young children with vaccinia virus had poor immune responses to the HIV-1 gp160 antigen compared with those who had no previous exposure to vaccinia virus. The results from this trial suggested that long-lasting immunity to vaccinia virus limited replication of the recombinant vaccinia virus used for immunization (6). The observations made during that study and data reported recently on the use of vaccinia virus recombinants expressing herpes simplex virus gene products in mice (8, 24) are consistent with our finding that vaccinia virus-specific memory T-cell responses are long lasting and may persist for life.

The results from the complement depletion assay using bulk-cultured cells demonstrated that $CD8^+$ memory CTL activity was dominant in short-term virus stimulated bulk cultures. We expected that the precursor frequency of the $CD8^+$

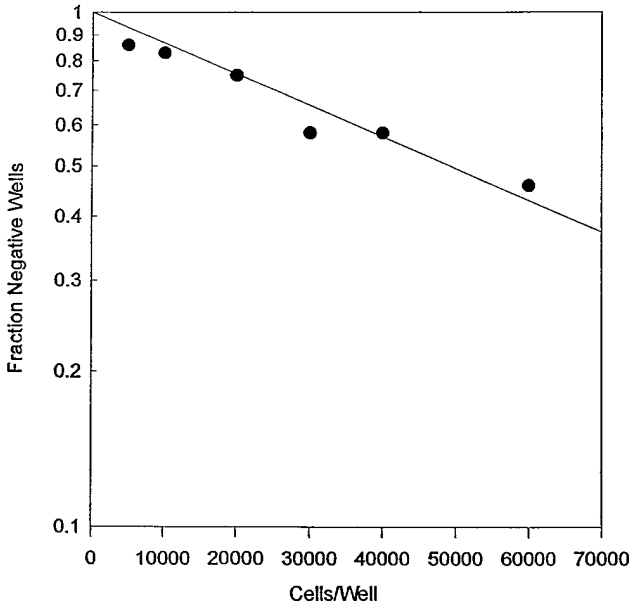

FIG. 1. Frequency of vaccinia virus-specific lytic effectors among sorted $CD4^+$ lymphocytes. Sorted $CD4^+$ T cells were stimulated with vaccinia virus antigen. Lytic activities were measured in a CTL assay on day 10. The frequency of vaccinia virus-specific $CD4^+$ CTL in donor VA15 was calculated as 1 in 65,920 $CD4^+$ T cells.

memory T-cell population would be greater than that observed for the $CD4^+$ T cells; however, we found it difficult to determine the precursor frequency of $CD8^+$ memory T cells. The number of $CD8^+$ cells isolated by fluorescence-activated cell sorter analysis was only one-third of the number of $CD4^+$ T cells, and our culture conditions with live virus may not have been optimal for detection of relatively low numbers of vaccinia virus-specific $CD8^+$ T cells.

The underlying mechanisms which contribute to immunological memory are poorly understood and have only recently received much attention. The major question with respect to persistent immunological T-cell memory is: how is it maintained in vivo? There is controversy about whether the maintenance of memory T cells requires periodic interaction with antigen-presenting cells expressing the relevant peptide (11, 22) or whether T-cell "memory" might be maintained in the absence of specific antigen stimulation (17, 20). Oehen et al. reported that adoptive transfer of immune spleen cells into syngeneic recipient mice required the presence of viral antigen for maintenance of the antiviral protective capacity of the transferred cells (22). Gray and Matzinger reported similar results (11). Other reports have challenged those findings (17, 20). Lau et al. used adoptive-transfer experiments in the lymphocytic choriomeningitis virus mouse model and reported that memory $CD8^+$ CTL persist and retain the memory phenotype indefinitely in the apparent absence of priming antigen, and these CTL apparently protected mice against virus challenge for up to 2 years (17). Mullbacher utilized a similar approach in a mouse model of influenza virus and also concluded that CTL memory is long-lived in the apparent absence of antigen (20). Our results obtained with PBMC of adult humans following immunization with vaccinia virus in early childhood also suggest that persistence of antigen is not required for long-term maintenance of T-cell memory. Although there is no evidence for the persistence of vaccinia virus or antigens in vivo, dendritic cells may sequester antigen for periods of time, making it available for persistent stimulation of the immune system (10).

If antigen persistence is not required, what other mechanisms contribute to long-lived, specific T-cell memory? One mechanism that has been suggested is immunological cross-reactivity between viruses (2, 21, 25). Selin et al. used percursor frequency analyses to study virus cross-reactive T-cell responses in mouse models and postulated that exposure to one virus might provide a boost in immunity to an unrelated virus (25). They could not rule out the possibility of enhanced nonspecific stimulation by lymphokines generated during the immune response to the heterologous virus, but their results tend to support the cross-reactivity hypothesis. In the absence of significant homology among unrelated viruses, they suggested that the observed T-cell cross reactivity may be due to cross-reactive epitopes possessing major amino acid differences but having discrete critical residues in common (25). This hypothesis may be reasonable in light of what is known about the phenotype of memory T cells (1, 9). When T cells acquire a memory phenotype, they upregulate the expression of several surface adhesion molecules in addition to the interleukin-2 receptor and become more sensitive to stimulation by a low-affinity, T-cell-specific peptide (21, 25). This "promiscuous" behavior may allow a memory T cell to become activated through an interaction of its T-cell receptor with an antigen-presenting cell presenting a peptide epitope from a virus unrelated to the virus that induced the original immune response. Shimojo et al. characterized a T-cell line generated against an influenza virus-encoded peptide which specifically recognized a dissimilar rotavirus-derived peptide (26), which supports this hypothesis. There is an increasing number of examples of immunological cross reactivity between proteins of infectious organisms and human proteins (23), and this molecular mimicry at the peptide level may play a role in T-cell cross reactivity in vivo. The interaction of T cells with antigen-presenting cells expressing self peptides which mimic peptide epitopes of infectious agents may also stimulate their propagation. Thus, memory T cells may be promiscuous in their ability to recognize various peptides and may be stimulated in a cross-reactive fashion.

The data presented here are perhaps the first clear evidence that virus-specific T-cell memory can persist for up to 50 years in humans in the presumed absence of antigen. We believe that human subjects with prior exposure to vaccinia virus years earlier provide an excellent model for the study of human T-cell memory. Further elucidation of the underlying mechanisms which contribute to the maintenance of T-cell memory will have an impact on our understanding of the basis of immunological memory and on the design of vaccines.

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Immunologic Responses to Vaccinia Vaccines Administered by Different Parenteral Routes

David J. McClain, Shannon Harrison,* Curtis L. Yeager,* John Cruz, Francis A. Ennis, Paul Gibbs, Michael S. Wright,* Peter L. Summers, James D. Arthur, Jess A. Graham*

Divisions of Virology and Medicine, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland; Brooke Army Medical Center, Ft. Sam Houston, San Antonio, TX; Division of Infectious Diseases and Immunology, Department of Medicine; University of Massachusetts Medical School, Worcester, MA

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Informed consent was obtained from human volunteers in accordance with human experimental guidelines of the U.S. Department of Health and Human Services and the Office of the Surgeon General of the U.S. Army (ref: AR 70-25).

The views, opinions, and/or findings contained herein are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation.

*Present affiliations: 465 Pine Dr., Jackson Hole, WY 83001 (S.H.); MCHJ-CI, Madigan Army Medical Center, Tacoma, WA 98431 (C.L.Y.); Abbott Diagnostic Division, Abbott Laboratories, 1921 Hurd Rd., Irving, TX 75015 (M.S.W.); Dept. of Radiology, Brooke Army Medical Center, Fort Sam Houston, San Antonio, TX 78234-6100 (J.A.G.)

Address correspondence (reprints) to: Dr. David J. McClain, Division of Virology, USAMRIID, Ft. Detrick, Frederick, MD 21702-5011

<Abstract>

To develop a less reactogenic but equally immunogenic vaccine, this study compared in 91 human volunteers the safety and immunogenic potency of a new, cell culture-derived vaccinia virus vaccine administered intradermally and intramuscularly to the licensed vaccinia vaccine administered by scarification. Cutaneous pox lesions developed in a higher proportion of scarification vaccinees. Scarification and intradermal vaccine recipients who developed cutaneous pox lesions had more local reactions, but also achieved significantly higher cell-mediated and neutralizing antibody responses than those who did not develop pox lesions. Although less reactogenic, intradermal or intramuscular administration of vaccinia vaccine without the concomitant development of a cutaneous pox lesion induced lower immune responses. Future studies will examine the cell-cultured vaccine's immunogenicity via scarification.

Vaccinia virus is a member of the Orthopox genus of the Poxvirus family with little virulence for immunocompetent humans. Apart from its critical role in the eradication of endemic smallpox, vaccinia virus has several biological properties that make it an excellent candidate for introducing foreign genes that prompted the investigation of recombinant vaccinia vaccines [1-3]. However, cellular immunity and antibody responses to vaccinia virus administered by different routes have not been compared in humans. These issues bear importance on how such vaccines will be used in humans, and perhaps why immune responses to recombinant vaccinia vaccines have required multiple injections [4-6].

The only vaccinia vaccine currently licensed in the U.S. was prepared from calf lymph and stored as a freeze-dried product. Supplies of this licensed vaccine cannot be replaced, as the production method for calf lymph vaccine is archaic and there is no adequate facility in which supplies can be regenerated in compliance with Good Manufacturing Practices. Certain U.S. military units may require smallpox vaccination during future deployments. Apart from military needs, the Centers for Disease Control (CDC) has recommended vaccination for persons working in the laboratory with vaccinia or recombinant vaccinia viruses [7]. Therefore, it is important that modern production techniques be applied to the manufacture of a vaccinia virus vaccine to make it safe and effective as a replacement for the current calf lymph smallpox vaccine. Given the attendant risks associated with the traditional method of cutaneous inoculation of vaccinia, we sought to evaluate the clinical and immune responses of alternative routes of inoculation by using a new, cell culture-derived, vaccinia vaccine.

The vaccinia vaccines used in the smallpox eradication effort were prepared on a large scale by inoculating the shaved abdomens of calves, sheep, or water buffalo with seed stocks of vaccinia virus, harvesting the infected exudative lymph from the inoculation sites, and bottling the product with phenol and brilliant green as bacteriostatic agents [8,9]. Partially because of the nature of this production in which bacterial contamination was expected, the vaccines were administered percutaneously with a bifurcated needle, a process that became known as scarification because of the permanent scar that resulted. Formation of a cutaneous pox lesion that healed with a scar formed an important method for verifying vaccination status during the era of endemic smallpox. This method proved effective and successful when applied by the World Health Organization to a campaign to globally eradicate smallpox [10].

As a consequence of this percutaneous inoculation, infectious vaccine virus is present in the local lesion after scarification. Subsequently, there were cases of inadvertent auto-inoculation and inoculation of susceptible vaccinee contacts. Additional complications included severe local spread of vaccine virus in individuals with chronic skin diseases [11]. Consequently, if it is possible to do so without loss of immunogenicity of the vaccine, it is desirable to administer vaccinia by a route that does not result in cutaneous lesions containing transmissible virus. Because of these safety concerns, human trials with recombinant vaccinia vaccines have used the injectable route.

We tested a new investigational vaccinia vaccine (BB-IND 4984) produced in cell culture. It was developed for parenteral injection to preclude the potential complication of inadvertent inoculation of virus attendant to scarification. During preclinical studies, this cell-cultured vaccinia vaccine candidate proved comparable to the New York Board of Health, Bureau of Biologics reference strain with respect to pock formation on chorioallantoic membranes of embryonated chicken eggs, formation of lesions after intradermal inoculation of adult rabbits, and intracerebral and intraperitoneal virulence in adult and suckling outbred mice (data not shown). A subsequent Phase I dose-escalation trial evaluated this vaccine in humans by subcutaneous inoculation. However, cutaneous vesicular (pox) lesions developed in an increasing number of volunteers as the subcutaneous dose was increased. Neutralizing antibody and lymphocyte proliferation assays indicated a higher and earlier immune response in vaccinees with cutaneous lesions than in vaccinees who did not develop pox lesions (DJ McClain, unpublished observations). Since the majority of pox lesions occurred in volunteers receiving the highest dose, confounding of pox lesion formation with dose could not be excluded in the statistical analysis. Therefore, we undertook a larger study tc examine whether formation of a cutaneous pox lesion was critical for optimal immune responses compared to injection of the cell-cultured vaccinia vaccine.

Methods

Vaccines. The licensed vaccinia (or smallpox) vaccine (Wyeth-Ayerst Laboratories, Philadelphia, PA) is a lyophilized vaccinia virus derived from the New York Board of Health strain [7], and is the only remaining licensed vaccinia vaccine in the U.S. The cell-cultured vaccinia vaccine was manufactured according to Good

Manufacturing Practices after three successive passages in MRC-5 cells. This vaccine lot was derived from the master seed of the smallpox vaccine previously licensed by Connaught. The Connaught vaccine was one of several licensed vaccinia products used in the U.S. until the end of smallpox vaccination in the early 1970s.

The cell culture-derived vaccinia vaccine was administered in a dose of 5.1 $\log_{10}$ plaque-forming units (PFU), either as 0.1 ml volume intradermally (ID) or 0.5 ml intramuscularly (IM). The licensed vaccinia vaccine was administered by scarification, as directed in the package insert, by dipping a sterile bifurcated needle into the vaccine and then pricking the skin inoculation site 3 times. The licensed vaccinia vaccine has a viral titer of approximately 10^8 PFU/ml, and the administration of 1 drop via scarification with a bifurcated needle is estimated to deliver 2.5 µl. Therefore, approximately 10^5 PFU is delivered by this method. All vaccinations were administered over the deltoid region of the arm.

Subjects. The vaccines were administered in an open-label study to healthy vaccinia-naive volunteers. Volunteers were screened by medical history and physical examination before enrolling in the study, with specific attention paid to the presence of a vaccinia vaccination scar or any contraindication to vaccination. Laboratory parameters before enrollment included HIV serology, serum chemistries, a complete blood count, and pregnancy test. Subjects were accepted if they were in good health, had no vaccinia scar or history of vaccination, and possessed no significant abnormalities that indicated an increased risk for vaccinia immunization, i.e. exfoliative skin disease or disorders of cellular immunity. Those criteria excluding a person from participating in the protocol were the same as those recommended by the CDC [7]. Volunteers who lacked a vaccination scar but subsequently proved to be vaccinia-immune by their baseline serologic test (50% plaque-reduction neutralization titer of $\geq$1:20) were excluded from statistical analyses.

Vaccinations and study design. Volunteers were randomized into 3 groups, with one-third of the volunteers receiving either licensed vaccinia vaccine by scarification, the cell culture-derived vaccinia vaccine IM, or the cell-cultured vaccinia vaccine ID. In addition, one-half of volunteers vaccinated ID were randomly selected to have their inoculation site wiped with alcohol immediately after injection. This procedure was to determine if immune recognition afforded by intradermal processing of antigen could be achieved without the formation of a cutaneous pox lesion. All volunteers vaccinated IM underwent alcohol wiping at the injection site postinoculation so as to

minimize the chance of accidental dermal inoculation with the vaccine. All inoculation sites were initially covered with a semipermeable dressing until any pox lesion had scabbed or until day 10 postinoculation (if no pox lesion developed). During the month following vaccination, subjects were seen as outpatients twice a week during the first 2 weeks postvaccination, and then weekly for the next 2 weeks. These outpatient examinations consisted of clinical examinations and laboratory tests to assess adverse reactions, potential complications, and immunogenicity.

Clinical assessments. Volunteers underwent semiweekly assessment for local signs or symptoms around the inoculation site as well as for potential systemic symptoms related to vaccination. Any local reaction at the inoculation site was measured to determine the diameter of any local erythema and induration, as well as scored for the presence or absence of warmth, tenderness, lymphadenopathy, subcutaneous nodule, or a vesicle (pox) lesion. Systemic reactions were assessed by questioning and examining the volunteers for the presence and severity of fever, chills, malaise, headache, myalgia, arthralgia, loss of appetite, nausea, vomiting, diarrhea, pruritus, or rash. These symptoms were quantitatively scored based upon severity (see table 1). Data were entered into a database for subsequent statistical analysis. The total local or systemic symptom score for a given vaccinee was defined as the sum of all scores for either local or systemic symptoms, respectively.

Clinical laboratories. Blood samples were obtained by weekly phlebotomy of volunteers beginning before vaccination until approximately one month postvaccination. A complete blood count with a 5-part differential count was performed by using a CellDyn 3000 (Abbott Laboratories, Chicago). Serum samples were analyzed by an Ektachem 700XR (Eastman Kodak, Rochester) for a panel of chemistry values (sodium, potassium, chloride, bicarbonate, urea nitrogen, creatinine, glucose, calcium, phosphorus, lactate dehydrogenase, aspartate transaminase, alanine transaminase, gamma glutamyl transferase, alkaline phosphatase, total bilirubin, and creatine phosphokinase). Test value means were calculated for each group on a given day of measurement. The data from each hematologic and serum chemistry test were analyzed using Statistical Analysis System (SAS version 6.10, Cary, NC) Procedure GLM repeated measures analysis of variance. The overall differences between groups over the course of the study period were compared by using the univariate tests of hypothesis for within subjects effects.

Serologic assays. Serum specimens from days 0, 10, 14, 20, and 27 were frozen for subsequent enzyme-linked immunosorbent antibody (ELISA) and plaque-reduction neutralization titer assay. An ELISA technique was

performed as previously described [12] to assay for antibodies reactive with cell lysate antigens from vaccinia virus-infected cells, with the modification of human sera as the test specimen and goat anti-human IgG as the detector antibody (Kirkegaard & Perry, Gaithersburg). Given a lower limit of detection of the assay at 1:100 test serum dilution, negative titers (i.e., O.D. comparable to background) were reported as equal to 1:50.

Plaque-reduction neutralization titer (PRNT), an in vitro test of serum's ability to neutralize the Wyeth strain of vaccinia virus, was determined using a modification of the method of Earley [13]. Briefly, each coded serum sample was incubated at 56°C for 30 minutes, then diluted 1:10 in Eagle's Minimum Essential Media (EMEM) containing 10% heat-inactivated fetal bovine serum (FBS). A suspension of vaccinia virus, calculated to yield a dose of approximately 40-100 PFU/0.1ml, was prepared in Hanks Balanced Salt Solution (HBSS) with 40 mM HEPES. Twofold dilutions of serum samples were then mixed 1:1 with 40-100 PFU of vaccinia virus suspension and incubated at 37°C for 1 h. After incubation, test samples and controls were inoculated onto monolayers of Vero cells in 12-well cell culture plates. After adsorption for 1 h at 37°C, each monolayer was overlaid with 1 ml of 0.5% agarose (FMC Bioproducts, Rockland, ME) containing HEPES-buffered Eagle's basal medium with Earle's salts, and 5% heat inactivated FBS. After incubation for 2 days at 37°C in a humidified atmosphere of 5% (vol/vol) carbon dioxide (CO_2), each monolayer was stained with 1 ml of the agarose overlay containing 0.167 mg/ml of neutral red. The plates were returned to the incubator for 24-36 h and the plaques were counted. The number of plaques for each test sample was entered into a computer program to determine by PRNT using probit analysis. The endpoint titer was the highest serum dilution demonstrating greater than 50% reduction in the number of plaques for the average dose.

Lymphocyte proliferation assays. Proliferative responses of peripheral blood mononuclear cells (PBMC) to live vaccinia virus and heat-inactivated antigen were tested as previously described [14,15]. Results were expressed as a stimulation index, derived by dividing counts in wells containing antigen from counts in wells without vaccinia antigen or virus,.

In situ ELISA viremia assay. Volunteers underwent periodic serum sampling within the first 2 weeks postinoculation, with an aliquot frozen at -70°C until later assay for vaccinia viremia. Serum was assayed for viremia by a modification of an in situ ELISA. Briefly, Vero cells grown in 24- or 96-well plates were inoculated with 0.1

ml (50 µl for the 96-well plates) of a serum sample. After adsorption for 1 h at 35°C, the 24-well plates were re-fed with 1 ml (100 µl) of EMEM containing 2% heat-inactivated FBS. Cultures were incubated at 35°C in a humidified atmosphere of 5% CO_2 for 4-6 days. Cultures were decanted and fixed with 1 ml (200 µl) of 10% formalin for 15 minutes at room temperature. Plates were incubated with 1 ml (200 µl) of HBSS containing 1% bovine serum albumin (BSA) for 30 min. The blocker buffer was removed and 0.5 ml (50 µl) of a 1:1000 dilution of vaccinia mouse hyperimmune ascitic fluid (ATCC, Rockville) or normal mouse ascitic fluid was added to the appropriate wells for 1 h at 35°C. After washing 3 times, 0.2 ml (35 µl) of a 1:2000 dilution of peroxidase-labeled anti-mouse IgG (Kirkegaard and Perry) was added to all wells for 1 h at 35°C. Plates were then washed 5 times, and incubated 30 min at 35°C with 0.5 ml (80 µl)/well of ABTS (2,2'-azino-di[3-ethyl-benzthiazoline sulfonate (6)]) substrate (Kirkegaard and Perry). Both test samples as well as positive and negative controls were tested in duplicate. A positive control was prepared using tenfold dilutions of vaccinia virus in negative antibody serum. Using these "spiked" serum samples, 2 wells were inoculated with each dilution. Two wells were also inoculated with normal (noninfectious) human serum as a negative control. Results were read visually or spectrophotometrically at 414 nm. Titers were calculated as tissue culture infectious dose 50% ($TCID_{50}$) values according to the method of Reed and Muench [16], or the highest specimen dilution with an optical density of 0.2 units over the negative controls.

Statistical methods. The PRNT and ELISA antibody responses were analyzed by variance with repeated measures followed by multiple comparisons for the study groups using the Tukey-Kramer adjustment for multiplicity [17]. The data from each hematologic and serum chemistry test as well as lymphocyte transformation assays were analyzed for each study group by using SAS Procedure GLM analysis of variance (ANOVA). The overall differences between groups over the course of the study period were compared by using the univariate tests of hypothesis for within subjects effects. All statistical tests were performed at the alpha = .05 level unless otherwise indicated.

Results

Subjects. Ninety-one volunteers participated in this Phase II study. Data from eight volunteers were

excluded from statistical analysis because of pre-existing vaccinia immunity as determined by baseline serologies. Of the remaining 83 subjects, there were 11 females and 72 males.

During the study, 3 volunteers developed medical problems which were judged as unrelated to the protocol: 1 volunteer developed folliculitis 1 week after inoculation; another developed lower extremity cellulitis 2 weeks postinoculation secondary to an infected leg laceration; and a third developed nausea, vomiting, and abdominal pain, which led to subsequent laparoscopic appendectomy on day 8 postinoculation, with normal appendiceal histopathology and eventual full recovery.

Clinical assessments. Analysis of variance (ANOVA) for vaccine effects at each week yielded no significant differences between the study groups' temperature profiles (temperature maximums recorded each week).

The ID vaccinees who received alcohol wiping postinoculation and the IM vaccinees did not significantly differ with respect to incidence of pox lesion (25% vs. 4.5 %, p=.141). However, failure to wipe with alcohol increased the incidence in the ID group to 62.5%, which was significantly higher then the IM group ($p = .0002$). This was significantly less than the incidence of pox lesions in the scarification group (96.6%), which was higher than all the other groups ($p < .0001$).

The scarification group experienced the highest incidence of local reactions, the IM group experienced the fewest, and the ID subgroups were intermediate and indistinguishable from each other. Local symptom analysis indicated highly significant statistical differences between the study groups using both ANOVA and non-parametric Wilcoxon analysis ($p < .0001$). There was a higher total symptom score for the scarification group (mean of 169.2) and a significantly lower score for the IM group (mean of 3.0). Mean total scores for ID vaccinees with and without alcohol wiping were 61.7 and 90.4, respectively. Additional analysis exclusive of the IM group revealed that the scarification group still differed from the ID vaccinees ($p < .0001$ by ANOVA or non-parametric). When the two ID subgroups were compared to each other, no differences were found in local symptom scores ($p = .215$ by ANOVA or $p = .406$ by non-parametric). When comparing local symptom scores between volunteers who did and did not develop cutaneous pox lesions, overall and weekly score differences were significant using the non-parametric Wilcoxon test ($p = .0001$).

Systemic symptom analysis indicated no significant differences in the study groups with respect to total

symptom scores either by ANOVA ($p = .352$) or by non-parametric Wilcoxon test ($p = .412$). However, higher mean systemic scores occurred 2 weeks postinoculation in those volunteers who developed a cutaneous pox lesion ($p = .038$).

All volunteers initially had their immunization site covered with a vapor-permeable surgical dressing until the time at which any pox lesions have scabbed. However, 4 volunteers with primary vesicles from their vaccination subsequently developed secondary pox lesions adjacent to the inoculation site underneath the dressing. We noted that the dressings were occlusive enough to accumulate perspiration underneath and around the inoculation site. Consequently, in the majority of volunteers with pox lesions, vesicular exudate accumulated under these dressings, despite frequent dressing changes. This virus-containing exudate seeped under the dressing, and covered areas much greater than that of the primary pox lesion, with subsequent secondary or auto-inoculation. Subsequently, a non-occlusive and smaller dressing (i.e, dry gauze) was applied over the inoculation sites for the remaining volunteers. These dressing changes were performed if there was a scheduled clinical check, the bandage became wet, or there were any visible signs of dried exudate on the exterior surface of the bandage. After the institution of the dry gauze dressings, no further incident of secondary pox lesions or large areas of adhesive contact dermatitis were observed.

Clinical laboratories. Laboratory findings were based on the analysis of the weekly mean for each subject for weeks -1, 0, 1, 2, 3, and 4. Due to some missing values when volunteers omitted scheduled phlebotomies, no repeated measures ANOVA could be performed. Hence, an ANOVA was done at each week. Only sporadic differences were detected for any posttreatment week, and are so noted in table 2.

Serologic and virologic assays. Plaque reduction neutralization titers were significantly greater for the scarification group than either the IM or ID groups beginning at day 13 postinoculation ($p = .0001$) using a repeated measures analysis of variance. Moreover, volunteers from either scarification or ID groups who developed cutaneous pox lesions had significantly higher neutralization titers than those without pox lesions in comparisons adjusted for pre-vaccination baseline. There was no significant difference between scarification or ID volunteers who had developed a cutaneous vesicle. However, there was a statistically insignificant trend toward higher titers in the scarification (Wyeth) recipients. These PRNT responses are illustrated in Fig. 1.

As illustrated in Fig. 2, vaccinia ELISA antibody responses were significantly higher for the scarification

group at day 27 postvaccination than either the ID groups or the IM groups ($p = .0001$). In contrast to PRNT responses, significantly higher ELISA titers were seen in scarification vaccinees when compared to ID recipients who formed a cutaneous pox lesion ($p < .001$). There was greater standard error in this assay in comparison to PRNT.

No viremia was detected in any volunteer.

Lymphocyte proliferation assays. Lymphocyte proliferation assays examined stimulation indices pre- and postvaccination to both live vaccinia virus and heat-inactivated antigen. For the heat-inactivated antigen, responses to scarification were significantly greater than those of either the IM group or the ID subgroup with alcohol wiping postinoculation ($p = .0258$), but not significantly different than the ID group without alcohol wiping ($p = 0.46$). With live vaccinia virus as the antigen, scarification vaccinees exhibited significantly higher stimulation indices ($p = .0037$) than did either IM or ID groups. Both higher indices and greater standard deviation were seen in the assay with heat-inactivated antigen in comparison to the live virus assay.

For volunteers who developed a pox lesion (whether ID or by scarification), the stimulation indices pre- and postvaccination were significantly greater than in vaccinees who did not develop a cutaneous pox lesion, whether assayed by using live virus or heat-inactivated antigen ($p = .0001$). There was no significant difference in lymphocyte stimulation responses of those who formed pox lesions from the licensed vaccinia vaccine via scarification versus those with pox lesions from the cell-cultured vaccinia vaccine. These mean lymphocyte responses for heat-inactivated antigen are illustrated in Figure 3.

Discussion

This human study compared the safety and immunogenicity of intradermal and intramuscular administration of a cell culture-derived vaccinia vaccine to the licensed vaccine administered by the traditional route of scarification. The use of injectable vaccinia vaccines is not a new idea. Between 1930 and 1975, at least 8 strains of vaccinia virus were developed for parenteral administration in order to decrease virulence [18]. All were attenuated when compared to standard lymph strains and some were possibly over-attenuated, producing low neutralizing antibody levels after primary and booster inoculations [19]. With the eradication of smallpox following closely upon initial

development of most of these vaccines, comparable safety and efficacy data were not obtained on a scale comparable to lymph vaccines. In addition, data about cell-mediated responses were not documented for these injectable products.

In this study, vaccination reactions were readily apparent from clinical observation alone. Extensive laboratory monitoring of vaccinees identified no specific safety concerns or issues about the routes of vaccine administration. There was no evidence of viremia in any volunteer. Serum chemistry or hematology value differences between study groups were sporadic and relatively small. Given the number of datapoints and comparisons in this study, these were likely to be Type I errors. As these statistical differences do not represent medically important differences in a contiguous time frame, we do not attribute them to causal events related to respective treatments.

The study indicated that, although not severe, local reactions corresponded to formation of a cutaneous pox lesion. As expected, pox lesion incidence was significantly higher in the scarification group than the other groups, as were local symptoms. In addition, higher systemic scores occurred 2 weeks postinoculation in those volunteers who developed a cutaneous pox lesion. Either vaccine dissimilarities or the route of vaccination could account for the symptomatic differences between scarification vaccinees and ID vaccinees with a pox lesion: impurities and animal proteins peculiar to calf lymph exudate could explain in part the reactivity of the licensed vaccinia vaccine, or scarification may permit greater viral replication than would intradermal inoculation. Given comparable results in preclinical tests and the fact that both vaccines studied derived from New York Board of Health strains, it is unlikely that the cell culture-derived vaccinia virus possesses enough sequence variation or intrinsic biological disparities from the calf lymph-derived Wyeth vaccine to account for such differences. The ID vaccinees who received alcohol wiping postinoculation and the IM vaccinees did not significantly differ with respect to incidence of pox lesions, but the latter group had significantly fewer local symptoms than the ID vaccinees, who in turn had fewer than the scarification vaccinees.

This study strongly indicated that, although less reactogenic, intramuscular administration of vaccinia vaccine at a dose of 10^5 PFU fails to induce an immune response comparable to that elicited by standard scarification. Although a higher dose of vaccinia virus might have been attempted intramuscularly, the WHO Smallpox Eradication

Campaign [10] and other clinical trials [18-21] had supported the safety of a parenteral dose of 10^5 PFU as used in this trial. In a Phase I dose-escalation human trial, the cell culture-derived vaccinia vaccine was used safely subcutaneously up to a dose of $10^{7.8}$ PFU, but with an attendant increased risk of a cutaneous pox lesion. Although the crossing of an additional tissue plane with intramuscular injection may have lessened this occurrence, we were concerned that intramuscular injection of a similar high dose of vaccinia virus might risk viremia.

The study also demonstrated that intradermal inoculation will not reliably avoid the formation of a cutaneous pox lesion. Phase I data had raised similar concerns regarding the subcutaneous route of inoculation. In a study by Connor et al. [21], typical Jennerian vesicles were seen in 4-9% of children vaccinated subcutaneously with 10^3, 10^4, or 10^5 PFU. Furthermore, it is apparent that the presence of such a lesion, although undesirable for safety reasons, is necessary for the most robust immune responses. By cleansing the inoculation site postinoculation, we sought to determine if immune recognition afforded by intradermal processing of antigen could be achieved without the safety risks attendant to a cutaneous pox lesion. This concept stemmed from a previous observation that the cutaneous vesicle was prevented after intradermal inoculation of vaccinia if the needle wound was immediately cleansed with alcohol [22]. These results indicate that replication of the virus in the skin, not merely exposure to antigen-presenting cells (i.e., Langerhans cells) in that location, is essential to generate optimal immune responses to vaccinia antigens.

Vaccinees who developed cutaneous pox lesions achieved significantly higher ELISA and neutralizing antibody responses. The poor responses to intradermal inoculation (in those volunteers without pox lesions) and to intramuscular injection confirm and expand upon earlier observations concerning injectable vaccinations in children [19]. The investigators of that study concluded that "... until more is known about the importance of neutralizing antibody in the immunity to smallpox, it would seem unwise to administer vaccine by the sub-cutaneous route..." [21]. This conclusion was based upon a) the relatively poor rates of seroconversion (67%), as measured by vaccinia-neutralizing antibodies, in children vaccinated subcutaneously compared to percutaneously vaccinated counterparts (87%); and b) the unimpressive neutralizing antibody responses upon successful percutaneous revaccination of children whose primary vaccination was subcutaneous.

In addition to antibody responses, cell-mediated responses were also superior in volunteers who developed pox lesions (whether by intradermal or scarification route). There was no significant difference in lymphocyte

stimulation responses of those who formed pox lesions from the licensed vaccinia vaccine by scarification versus those with pox lesions from the cell culture-derived vaccinia vaccine. Inferior cell-mediated responses of vaccination without a pox lesion, as observed in this study, may explain why Connor et al. reported that children who had initially received an injectable (subcutaneous) vaccine exhibited >30% incidence of "primary-type" responses to percutaneous revaccination [21]. This study also confims the observation by Cherry et al. [20] of a correlation between neutralizing antibody and lymphocyte stimulation responsiveness to vaccinia after vaccination.

An unexpected event in this phase II study was the high incidence of secondary pox lesions adjacent to the inoculation site in the first set of volunteers (4 of 31). The use of semipermeable dressings appeared to have caused this complication, as no further instances occurred after instituting the use of dry gauze dressings. Although vapor-permeable, the semipermeable dressings caused accumulation of perspiration around the inoculation site despite frequent dressing changes, permitting virus-containing exudate to secondarily inoculate areas proximate to the primary pox lesion. The large adhesive area of this dressing caused various degrees of contact dermatitis in several volunteers, but this did not trigger auto-inoculation, since the secondary lesions were under the dressing area and not at the point of adhesive contact. Ironically, the use of these semipermeable dressings was originally mandated as a protective measure against contact and environmental spread of the vaccine virus. Apart from the four instances of auto-inoculation precipitated by semipermeable dressings, there were no serious or unexpected complications from the study; all inoculation site lesions healed completely.

If it is necessary to achieve immunity to vaccinia or related *Orthopoxviridae* such as variola or monkeypox, these results warrant vaccination by scarification in a population without contraindications, despite the attendant risks. Formation of a cutaneous pox lesion (i.e., a "take"), although less desirable from a safety standpoint, engenders higher PRNT titers and cell-mediated responses, and has been historically validated as indicating protective immunity [10]. This observation holds true when comparing intradermal or intramuscular administration of the cell culture-derived vaccinia vaccine when such does *not* result in the development of a cutaneous pox lesion. These data appear to reflect the epithelial tropism of Orthopoxviridae, which may explain many of the disappointing immune responses in humans to vaccinia-vectored gene inserts [23,24]. In addition, it remains dubious whether the greater risk of high-dose (e.g., 10^7 PFU) intramuscular inoculation would be warranted in an effort to achieve higher PRNT responses

without a cutaneous pox lesion, despite evidence from both preclinical and clinical studies that the cell culture-derived vaccinia vaccine is biologically comparable to other New York Board of Health strains. Although multiple injections of vaccinia via a parenteral route might improve immune responses closer to those seen with scarification, repeated injections would increase costs and logistical burdens and thereby negate the typical advantages of a live vaccine, yet with greater safety concerns.

These results have important implications for the use of recombinant poxvirus vaccines as vectors for immunogens, and may explain why immune responses to some of these vaccines have required multiple injections [4-6]. Although deliberate inoculation by scarification may provide improved immune responses for vaccinia virus-vectored immunogens, this is with a greater risk of local reactions and possible secondary inoculation. This rationale would not apply to highly attenuated poxvirus vectors which are incapable of productive replication in human-derived cell lines [25,26].

When intradermal administration of the cell-cultured vaccinia resulted in a cutaneous pox lesion, immune responses were not significantly different than those seen with scarification of the licensed vaccinia vaccine. Although there is a statistically insignificant trend towards higher neutralization titers with the latter, future studies will examine the cell-cultured vaccine's immunogenicity by this same scarification route.

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Figure 1. Vaccinia plaque-reduction neutralization titer (PRNT) 50% responses as illustrated by geometric mean titers (GMT). PRNT were significantly higher for scarification inoculees on repeated measures analysis of variance ($p = .0001$), but not significantly higher than intradermal recipients who developed a cutaneous pox lesion (ID w/ Pox) postvaccination ($p = .09$). ID w/o Pox = intradermal recipients without a pox lesion post-vaccination; IM = intramuscular recipients.

Figure 2. Enzyme-linked immunosorbent assay (ELISA) responses to vaccinia-infected whole cell lysates as illustrated by geometric mean titers (GMT). ELISA titers were significantly higher for scarification inoculees on repeated measures analysis of variance ($p = .0001$), even in comparison to intradermal (ID) recipients who formed a cutaneous pox lesion ($p < .001$). ID w/o Pox = intradermal recipients without a pox lesion post-vaccination; IM = intramuscular recipients.

Figure 3. Lymphocyte proliferation transformation responses to heat-inactivated vaccinia antigen. Stimulation indices were significantly higher for scarification inoculees on analysis of variance ($p = .0258$), but not significantly higher than intradermal (ID) recipients who developed a cutaneous pox lesion postvaccination ($p = .46$). ID w/o Pox = intradermal recipients without a pox lesion post-vaccination; IM = intramuscular recipients.

Table 1. Scoring for systemic symptoms and signs

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0 = No Symptom

1 = Mild (symptom can be ignored)

2 = Moderate (symptom affect activity/relieved by analgesics)

3 = Severe (symptom not relieved by analgesics)

Table 2. Statistically significant differences in laboratory values

Test	Study week	ID w/ wipe	ID w/o wipe	IM	SCAR	p-value
creatinine (mg%)	1	1.2	1.08	1.12	1.18	.0175
potassium (mEq/L)	3	4.2	4.49	4.25	4.49	.0043
phosphorus (mEq/L)	4	4.05	4.09	4.57	4.45	.0274
% monocytes	2	7.23	8.53	7.6	9.34	.0032
% monocytes	1	6.16	8.1	8.24	7.54	.0392

Data were analyzed for each study group by using SAS Procedure GLM analysis of variance (ANOVA). The overall differences between groups over the course of the study period were compared by using the univariate tests of hypothesis for within subjects effects (alpha = .05). Monocytes are expressed as the percentage of total white cells in the complete blood count. ID w/ wipe = intradermal inoculation with local alcohol wiping postinoculation; ID w/o wipe = intradermal inoculation *without* alcohol wiping postinoculation; IM = intramuscular inoculation; SCAR = scarification inoculation.

Figure 1

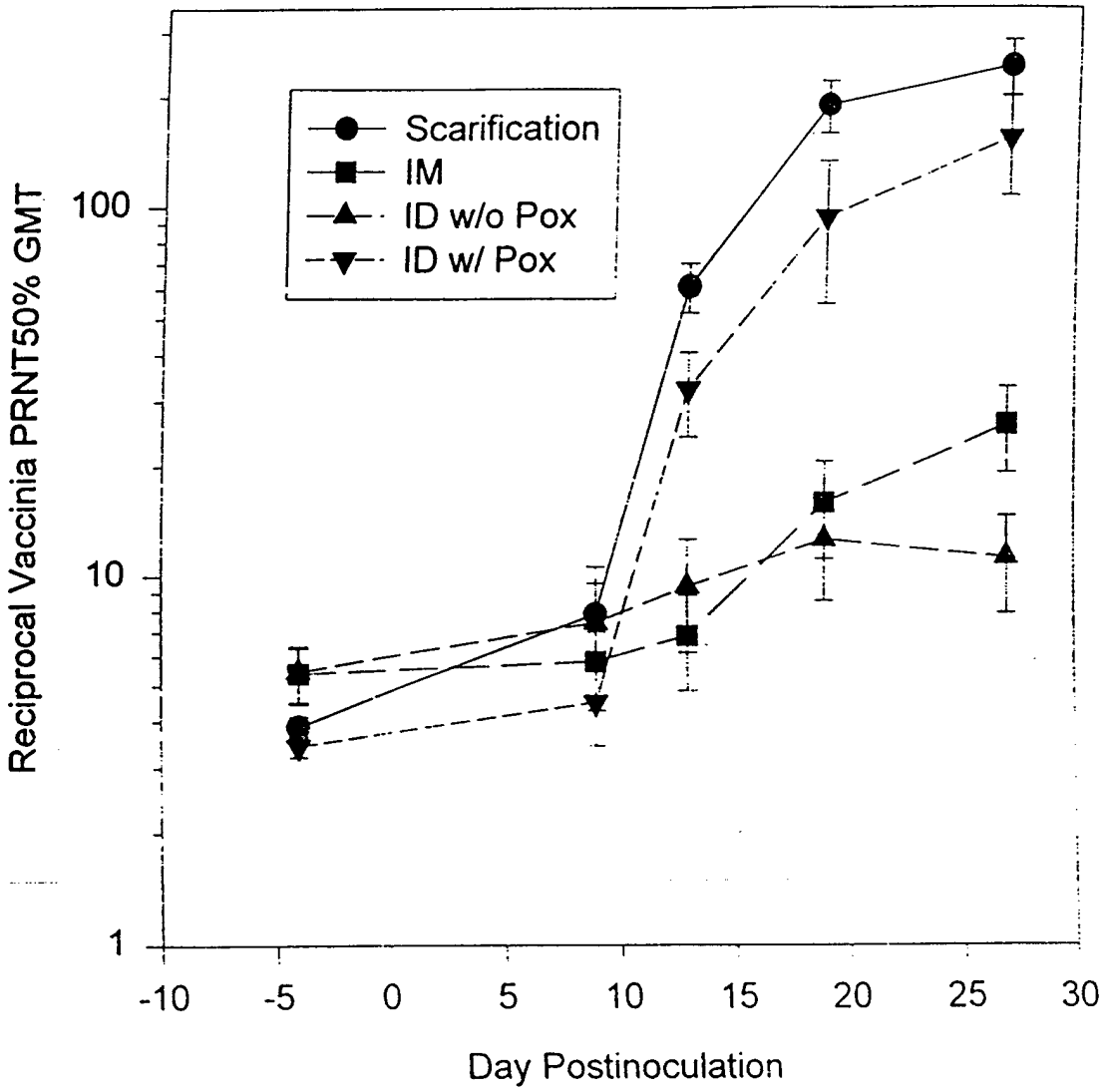

Scarification
IM
ID w/o Pox
ID w/ Pox
Reciprocal Vaccinia PRNT50% GMT
100
10
1
-10
-5
0
5
10
15
20
25
30
Day Postinoculation

Figure 2

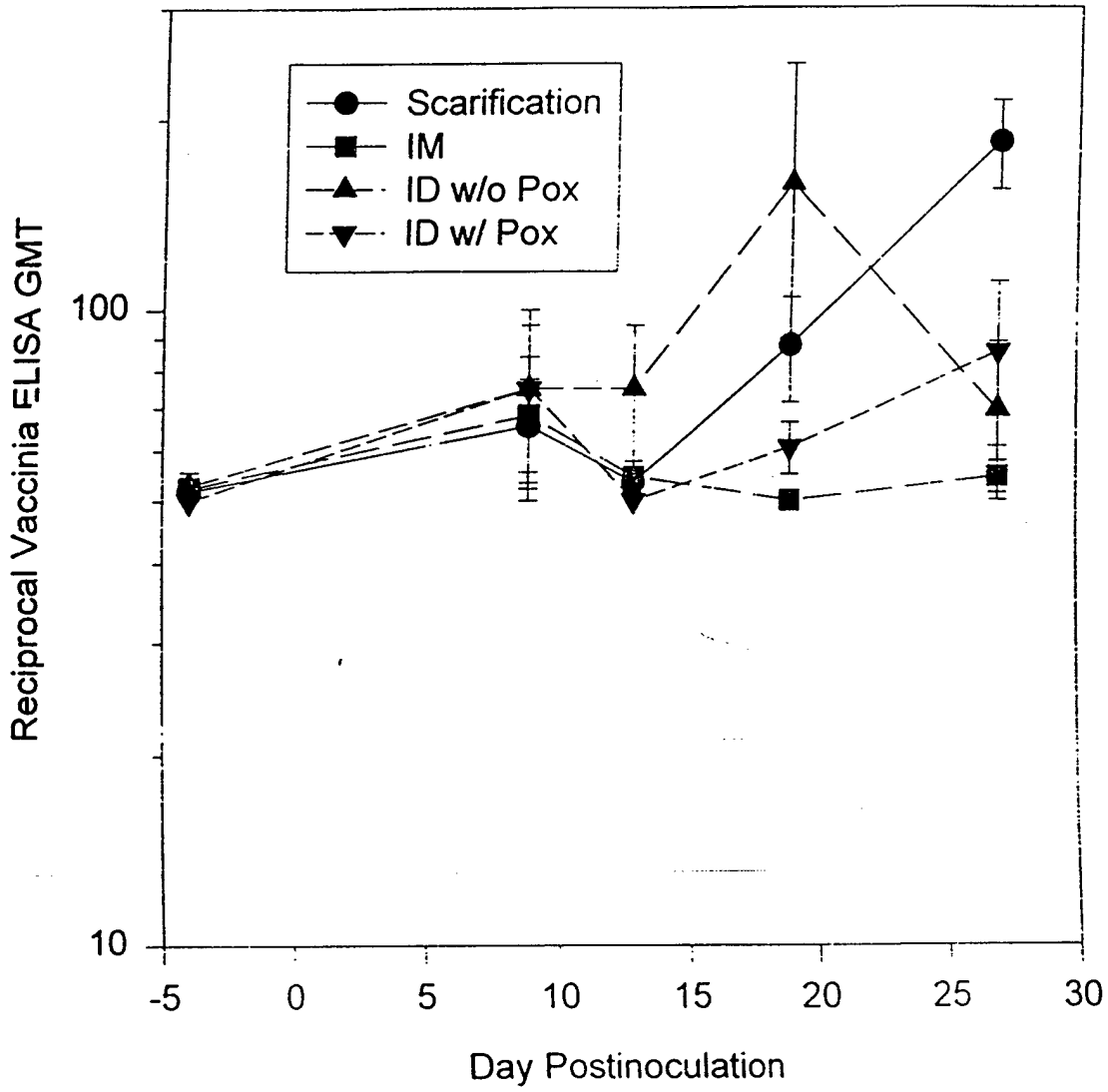
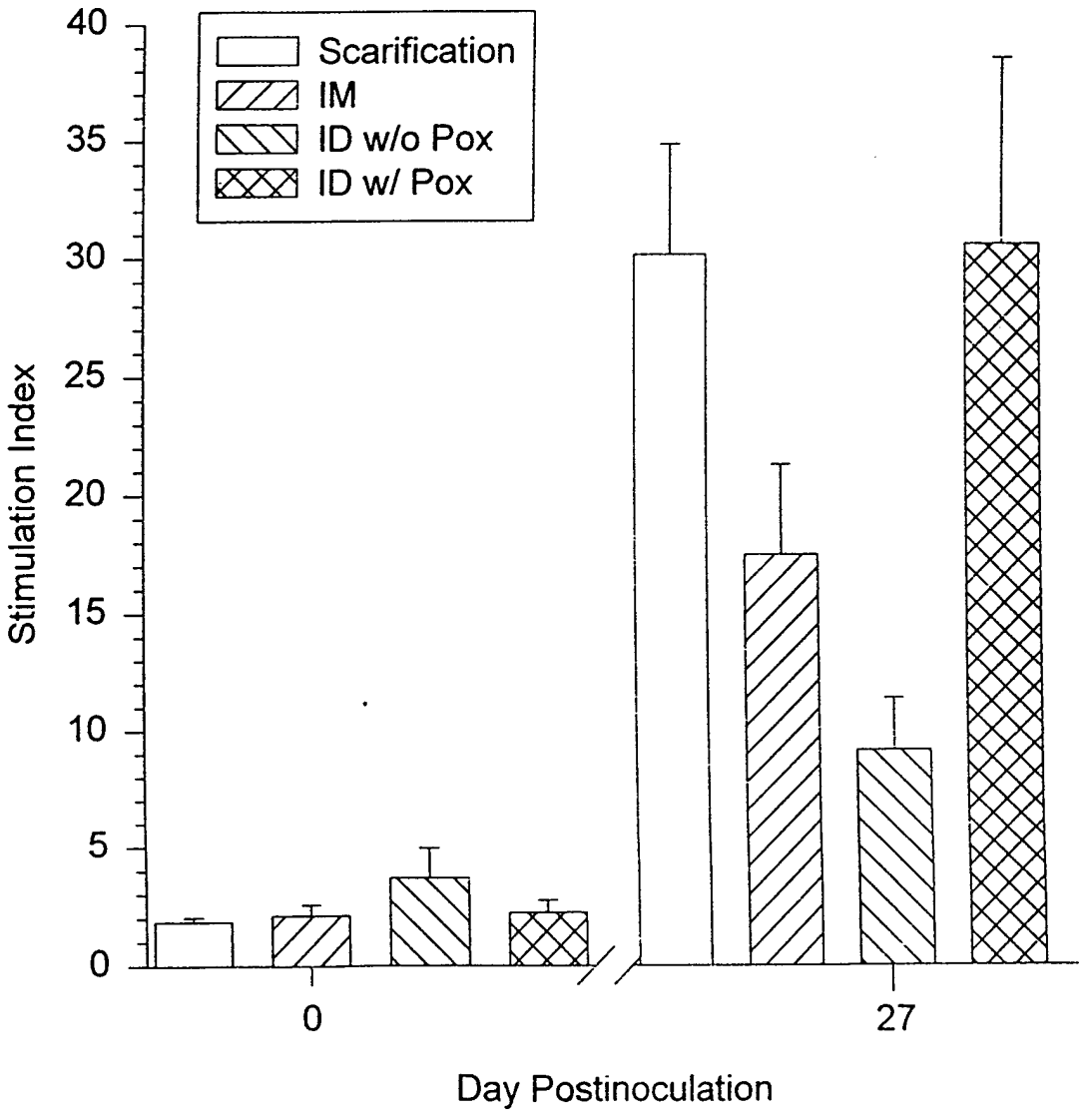

Scarification
IM
ID w/o Pox
ID w/ Pox
Reciprocal Vaccinia ELISA GMT
100
10
-5
0
5
10
15
20
25
30
Day Postinoculation

Figure 3


Scarification
IM
ID w/o Pox
ID w/ Pox
Stimulation Index
40
35
30
25
20
15
10
5
0
0
27
Day Postinoculation