Study of Immune Function in Inbred Miniature Pigs Vaccinated and Challenged with Suid Herpesvirus 1

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ABSTRACT

Specific immune responses of inbred miniature pigs following vaccination and challenge with suid herpesvirus 1 (SHV-1) were determined. Vaccination of swine with SHV-1 elicited both specific neutralizing antibody and lymphoproliferative responses. Moreover, pigs vaccinated with SHV-1 were fully protected against a lethal virus challenge. Pigs vaccinated with a recombinant (r) SHV-1 virus, followed by challenge with a virulent SHV-1, had lower percentages of circulating T- and B-lymphocytes, and showed a significant ($P \leq 0.05$) reduction in peripheral blood mononuclear cell (PBMC) antibody-dependent cell-cytotoxicity than control (noninfected, SHV-1 seronegative) animals. From the 5th through the 8th week of postchallenge, rSHV-1 was isolated from 2 of 4 pigs. Presence of r-virus was indicative that PBMC were infectious in vivo. The rSHV-1, with beta-galactosidase activity, was only recovered from ConA- and IL-2-stimulated primary PBMC cocultivated with porcine kidney cells. Control pigs exposed to challenge SHV-1 elicited both specific neutralizing antibody and lymphoproliferative responses followed by subsequent infection. These infected pigs, compared to control pigs, had significantly ($P \leq 0.05$) lowered percentages of T- and B-lymphocytes, lowered T-cell mitogenic responses, variable PBMC counts, and lowered blood phagocytic cell function. When PBMC from control pigs were cultured and infected with SHV-1, the virus caused a significant ($P \leq 0.05$) suppression of T-cell proliferation and PBMC mitochondrial dehydrogenase and macrophage activities.

RÉSUMÉ

Suite à la vaccination de porcs miniatures avec le virus herpès porcin de type 1 (SHV-1), des anticorps neutralisants spécifiques et une réponse de type lymphoproliférative ont été détectés. De plus, ces animaux ont résisté à une infection expérimentale mortelle. En comparaison à des animaux témoins (non infectés, séro-négatif pour SHV-1), les porcs vaccinés à l'aide d'un virus SHV-1 recombinant (rSHV-1) puis infectés avec un isolat virulent de SHV-1 avaient relativement moins de lymphocytes circulants de type T et B et une réduction significative ($P \leq 0.05$) du nombre de mononucléaires cytotoxiques (PMBC). De la cinquième à la huitième semaine suivant l'infection, il a été possible d'isoler le rSHV-1 à partir de deux des quatre porcs. La présence de virus recombinant indiquait qu' in vivo les PMBC étaient encore infectieux. Le rSHV-1 n'a pu être réisolé que des PMBC stimulés à la ConA et IL-2 en co-culture avec des cellules rénales de porc. Chez des animaux témoins exposés au SHV-1 on a noté l'apparition d'anticorps neutralisants spécifiques et d'une réponse de type lymphoproliférative suivi d'une infection apparente. En comparaison aux témoins négatifs, ces animaux avaient des niveaux significativement plus bas de lymphocytes T et B, une réponse aux mitogènes plus faible, des comptes variables de PMBC et une diminution de fonction des phagocytes sanguins. Une inhibition significative ($P \leq 0.05$) de la prolifération des lymphocytes T, de la déshydrogénase mitochondriale des PMBC et des fonctions des macrophages ont été notées lorsque les PMBC des témoins négatifs ont été mis en culture et infectés avec le SHV-1.

(Traduit par Docteur Serge Messier)

INTRODUCTION

Aujeszky's disease, or pseudorabies, is caused by suid herpesvirus 1 (SHV-1), an alphaherpesvirus. The disease is characterized by nonsuppurative meningoencephalitis, pneumonia, reproductive failures, and high mortality in piglets under 8 wk of age. In adult pigs, SHV-1 induces sporadic disease with relatively low mortality. Pigs frequently survive the viral infection and subsequently become an important reservoir of infection. In the past 2 decades, a significant increase in the emergence of more virulent strains of SHV-1 has occurred in swine production herds within the United States of America (1).

The pathogenesis of SHV-1 in pigs involves the respiratory tract as a primary site of entry. The virus replicates in the nasal and oropharyngeal mucosa, tonsillar tissues, and the upper respiratory tract, and then invades the central nervous system and lower respiratory tract. The infection extends from the primary sites of replication towards draining lymph nodes (1). Given the possibility of a hematogenic route for SHV-1 dissemination, the role of peripheral blood mononuclear cells (PBMC) becomes important. These cells participate in

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TABLE I. Distribution of peripheral blood leukocytes in SHV-1 seronegative pigs identified as Group I, vaccinated with killed virus (wk 0) and challenged (wk 4) and Group II, exposed to a recombinant virus (wk 0) and challenged (wk 4 and 8)

Time (week)	Pig Group I[a] Killed SHV-1 and Virulent SHV-1				Pig Group II[a] Live rSHV-1 and Virulent SHV-1			
	WBC	PMN	% T	% B	WBC	PMN	% T	% B
	SHV-1 seronegative pigs, $n = 7$				SHV-1 seronegative pigs, $n = 7$			
−3	13 ± 0.8	6.8 ± 0.6	58 ± 14	31 ± 7	13 ± 0.8	6.2 ± 0.5	57 ± 12	31 ± 7
−2	13 ± 0.9	6.9 ± 0.5	56 ± 12	30 ± 6	12 ± 1.2	6.9 ± 0.6	59 ± 13	26 ± 5
−1	12 ± 1.5	6.7 ± 0.7	53 ± 8	28 ± 7	14 ± 1.4	7.1 ± 0.6	56 ± 12	29 ± 6
0	11 ± 1.9	6.6 ± 0.8	51 ± 11	29 ± 6	12 ± 1.1	6.9 ± 0.8	57 ± 15	30 ± 6
	IM-vaccinated with killed virus, $n = 6$[b]				IN-exposed to live avirulent rSHV-1, $n = 6$[c]			
1	12 ± 0.7	6.8 ± 0.8	49 ± 12	29 ± 5	13 ± 0.9	6.6 ± 0.6	52 ± 13	34 ± 8
2	13 ± 0.9	6.8 ± 0.7	48 ± 12	30 ± 8	12 ± 1.1	5.9 ± 0.7	43 ± 9	24 ± 7
3	12 ± 1.2	6.4 ± 0.9	45 ± 11	27 ± 9	14 ± 1.8	5.9 ± 0.7	43 ± 9	29 ± 8
4[d]	13 ± 1.1	6.3 ± 1.1	47 ± 9	30 ± 9	12 ± 1.0	6.7 ± 0.9	48 ± 10	28 ± 8
	IN-exposed to live virulent virus, $n = 4$[d]				IN-exposed to live virulent virus, $n = 4$[d]			
5	13 ± 0.9	6.5 ± 0.8	41 ± 12	28 ± 8	11 ± 1.2	7.8 ± 0.7	44 ± 12	19 ± 6[e]
6	12 ± 1.3	6.8 ± 1.1	35 ± 10[e]	22 ± 9[e]	10 ± 1.4	5.9 ± 0.9	34 ± 14[e]	21 ± 7[e]
7					13 ± 0.9	7.1 ± 0.7	43 ± 10	26 ± 8
8					14 ± 1.1	7.2 ± 0.8	42 ± 11	25 ± 10
					IN-reexposed to live virulent virus, $n = 2$[d]			
9					12 ± 1.3	6.2 ± 0.9	42 ± 13	26 ± 9
10					12 ± 1.9	6.3 ± 1.1	40 ± 15	25 ± 11

[a] Computed as geometric means of white blood (WBC) and polymorphonuclear (PMN) cell counts ± SD × 10^6/mL of EDTA-treated blood. Percentages of T- and B-lymphocytes were based on E and EAC rosettes

[b] Killed SHV-1 vaccine administered (1.0 mL/pig) intramuscularly (IM) on d 1

[c] rSHV-1 intranasally (IN) administered in 1.0 ml (1.4 × 10^7 $TCID_{50}$)/pig on d 1

[d] SHV-1 (Strain P-2208) IN administered in 1.0 ml (1.2 × 10^8 $TCID_{50}$)/pig on the 28th d of groups I and II, and also on the 56th d of group II

[e] Significantly ($P \leq 0.05$) lower numbers of respective cells were observed following SHV-1 challenge, compared to numbers of respective cells obtained from SHV-1 seronegative pigs

cell-mediated immune (CMI) functions against SHV-1-infected tissues (2–4). Also, alveolar macrophages, infected with SHV-1, may reenter the blood stream and disseminate infection (5).

The goal of this study was to determine the effects of SHV-1 challenge on specific immune responses of inbred miniature pigs following SHV-1 vaccination. Experiments were factorially designed to compare control, killed- and modified-live-vaccinated, and live-virus-exposed pigs by measuring blood cell differential counts, PBMC functions in the presence of cytokines, SHV-1 antibody, and antigen-specific lymphocyte responses. PBMC activities and recovery of SHV-1 were used to determine the incidence of SHV-1 infections. At postmortem, spleen cells were collected from infected and non-infected pigs. These cells were assayed for SHV-1 antibody-dependent cell-cytotoxicity (CC) responses. Immunity to SHV-1 includes both neutralizing antibody and CMI, and protection against clinical disease can be ensured by vaccination (1). Vaccines currently available, however, may reduce, but do not prevent, virus excretion or establishment of latency after infection.

MATERIALS AND METHODS

ANIMALS

SHV-1 seronegative Minnesota miniature pigs, ≥6 mo of age, were used in this study (6). Such pigs were earlier developed by a selective inbreeding at the major histocompatibility complex (MHC), or swine leukocyte antigen (SLA) complex scheme, based on tissue typing of the offspring of each generation; SLA haplotypes aa, ad, cc; Iowa State University, Madrid, Iowa). They were randomly divided into experimental groups (Table I). Group I animals were vaccinated with killed SHV-1 and then challenged with virulent SHV-1. Group II animals were exposed to live avirulent recombinant (r) SHV-1, then challenged twice with virulent SHV-1. Care of the pigs was according to the guidelines for the use and care of laboratory animals provided by the National Institutes of Health. Blood was collected aseptically by brachiocephalic vein puncture and dispensed into sterile serum collection and preservative-free EDTA vacutainer tubes (7).

VIRUSES

Viruses administered to pigs are shown in Table I. A recombinant (r) avirulent strain of SHV-1 (prepared from Iowa S-62 strain with gene deletion: TK, gpX; with a titer of 1.4 × 10^7 median tissue culture infectious doses, $TCID_{50}$/mL; Syntrovet, Lenexa, Kansas) was administered intranasally (IN). A virulent P-2208 (Indiana, 1.2 × 10^8 ($TCID_{50}$)/mL) strain of SHV-1 was also administered IN. A killed SHV-1 vaccine preparation (SmithKline Beecham Animal Health, Lincoln, Nebraska) was administered intramuscularly (IM).

BLOOD

Time lines of immunization and challenge protocols are presented in Table I. Blood from individual animals was collected weekly, sedimented with a 1/10 volume of 0.6% Dextran T-500 and the plasma separated on Ficoll-Hypaque (density 1.077, Pharmacia, Piscataway, New Jersey) for 30 min at 400 × *g*. PBMCs at the interphase were washed with Hanks' balanced salt solution, and resuspended in RPMI-1640 medium (GIBCO, Grand Island, New York) supplemented with 10% fetal bovine serum (FBS), 1 mM L-glutamine, and penicillin (100 U/mL), streptomycin (100 ug/mL), and gentamicin (100 ug/mL) [RPMI plus supplement (RS)-medium]. The cells were incubated, with and without culture additives (see below) in duplicate flatbottom 96-well microtiter plates at a concentration of 5 × 10^5 cells/well (8). Following 48 h incubation with and without culture additives (see below), luCi/well of methyl-^{3}H-thymidine (spec. act 49 Ci/mmol) was added for 24 h, the cells were then harvested onto fiberglass filters, and counted in a scintillation counter.

PBMC were also incubated in plastic flasks for 1 h in the presence of carbonyl iron (0.1 mg/mL). Following

retention of macrophages by a selenium cobalt magnet, the PBMC were separated into subpopulations using an immunopanning procedure. This procedure consisted of dispensing the cells in dishes coated with purified rabbit antiswine IgG (United States Biochemical Corp., Cleveland, Ohio) antibodies for 1 h at 5°C. Afterwards, the nonadherent cells were collected and identified as consisting of T-lymphocytes and monocytes. The adherent cells, consisting of enriched B-lymphocytes, were washed and mechanically detached after an incubation in phosphate buffered saline solution with 10% porcine serum without anti-SHV-1 antibodies for 90 min at 37°C. For differentiation of T- and B-cells, the erythrocyte-antibody-complement (EAC) and E (erythrocyte) methods were used (9,10). Sheep erythrocytes were mixed with an equal volume of porcine lymphocytes. The mixture was centrifuged at 50 × *g* for 5 min and incubated at 4°C overnight. Following centrifugation, the cells were resuspended and counted for T-cells according to number of E rosettes per 100 lymphocytes. For determining B-cell numbers, computations were based on one B lymphocyte binding ≥2 EAC rosettes (Table I). Mean values for lymphocytes were compared to values obtained from weekly bleedings from 14 SHV-1 seronegative pigs.

TABLE II. Proliferative responses of porcine ConA-stimulated PBMC cultures incubated with cytokines and SHV-1[a]

Culture Additive[b]			Treatment Groups of Pigs Mean stimulation indices (SI) ± SD		
IL-1	IL-2	LAP	[seronegative] Control Week 0	[vaccinated] Killed SHV-1 Week 4	[exposed to live virus] rSHV-1/SHV-1 Week 6
−	−	−	2.3 ± 0.33	2.9 ± 0.26	2.2 ± 0.27
+	−	−	2.4 ± 0.35[c]	4.1 ± 0.42[d]	1.9 ± 0.63
+	+	−	4.6 ± 0.67[cd]	7.1 ± 1.01[d]	1.8 ± 0.44
+	+	+	3.6 ± 0.58[d]	4.6 ± 0.73[d]	1.6 ± 0.57
−	+	−	5.7 ± 0.84[d]	7.2 ± 1.23[d]	1.8 ± 0.47
−	+	+	5.1 ± 0.81[d]	6.3 ± 0.92[d]	1.8 ± 0.46
−	−	+	1.4 ± 0.31	1.8 ± 0.41	1.8 ± 0.46

[a] SHV-1 (strain P-2208) was incubated at an MOI of 1.0 with PBMC cultures, representing all treatment groups (Table I). SI were computed based on uptake of m³Ht, equal to the ratio of experimental cpm to mock cpm

[b] Cytokines were added (+) or deleted (−) initially to ConA (5 ug/mL)-stimulated PBMC at concentrations of: IL-1, 1.0 ug/mL, IL-2, 64 U/mL, LAP, 10 ug/mL (alone and in combinations) and remained during 66 h incubation. The latter 18 h the PBMC cultures were incubated with m³Ht. Results are from six replicates/animal, four pigs/group. PBMC cultures (mock) incubated with m³Ht, without culture additivies, incorporated a mean radioactivity of $1.9 \pm 0.7 \times 10^{-3}$ cpm

[c] SI were significantly ($P \leq 0.05$) lower for PBMC cultures from seronegative pigs than for PBMC cultures from killed SHV-1 vaccinated pigs incubated with either IL-1 or combination of IL-1 and IL-2

[d] SI were significantly ($P \leq 0.05$) higher for PBMC cultures from control and killed SHV-1 pigs than for PBMC cultures from pigs exposed to live virus

CULTURE ADDITIVES

Concanavalin A (ConA) and human interleukins 1 and 2 (IL-1, IL-2), and lymphocyte-activating pentapeptide (LAP, mw 568.7; sequence position 351-355 in the Fc region of human IgG), were dissolved separately in RS-medium. These culture additives were purchased from Calbiochem Biochemicals (San Diego, California). Concentrations of additives used are given in the legend of Table II. Rationale for using these additives as enhancers of PBMC proliferations has been discussed previously (8).

PROLIFERATIVE STUDIES OF PBMC

Phytomitogen (ConA), SHV-1, and cytokines (IL-1, IL-2, LSP) were added directly to triplicate wells upon initiation of PBMC cultures (Table II) (7). SHV-1 was added after the initiation of PBMC cultures with a given mitogen and/or cytokine. Cultures were incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2-95% air and then incubated with 0.5 uCi of methyl-[³H]-thymidine (m³Ht) for 18 h. Cultures were harvested onto glass-wool filter discs, dispensed in scintillation fluid, and counted for radioactivity. All cultures were performed in 6 replicate wells per test. Results were expressed as mean counts per min (cpm) that were then computed as stimulation indices (SI; Table II).

DETECTION OF SHV-1 IN CULTURES

PBMC were collected from SHV-1-infected pigs, and processed as above. The PBMC were incubated at 37°C for 48 h in RS-medium with ConA (10 ug/mL), IL-2 (64 U/mL) and gliotoxin (1 ug/mL, extracted from *Aspergillis fumigatus* by Dr. John Richard, NADC, Ames, Iowa). The PBMC were collected by centrifugation (300 × *g* for 15 min). Cells were seeded (about 1×10^6/well) on confluent porcine kidney (PK_{15}) cells in 24-well microtiter plates containing minimal essential medium (MEM, see below). The cultures were incubated, as above, and were observed daily for cytopathic effects (CPE). Subpassages were made at the end of 7 d incubation. All cultures were observed for 21 d. Cultures were assayed for rSHV-1 beta-galactosidase activity using Bluogal (halogenated indolyl-beta-D galactoside; 0.7 mg/mL RS-medium; Life Technologies, Inc., Gaithersburg, Maryland) as substrate, and for presence of SHV-1 positive cells by reaction with specific fluorescein isothiocyanate, see below.

IMMUNOLOGICAL METHODS IN CULTURES

Cell cultures and culture supernatants were tested for the presence of SHV-1 using hyperimmunized porcine polyvalent anti-SHV-1 serum labelled with fluorescein isothiocyanate (from E.C. Pirtle; 11). Confluent monolayers of PK_{15} cells were incubated with SHV-1 or culture supernatants. After 24 h incubation, the cells were treated with cold acetone-methanol, and incubated for 60 min with the above antiserum, diluted 1:16 and 1:32. Cells were then washed 3 times with phosphate-buffered saline (PBS, pH 7.1), and observed for fluorescent cells by ultraviolet light microscopy.

Pig sera were heat-inactivated (56°C for 30 min), and serially two-fold diluted. Presence of neutralizing SHV-1 antibodies in pig sera were detected by a standardized microtiter virus neutralization (VN) test (12), a latex agglutination (LA) test kit (Viral

TABLE III. Effect of SHV-1 on PBMC mitochondrial (MTT) and phagocytic (CL) activities of PBL from control-, killed SHV-1 vaccinated-, and rSHV-1/virulent SHV-1 challenged- pigs[a]

Culture Additive	Test	Treatment Groups of Pigs Mean stimulation indices (SI) ± SD (log_{10})[a] [seronegative] Control Week 0	[vaccinated] Killed SHV-1 Week 4	[exposed to live virus] rSHV-1 Week 4	[exposed to live virus] rSHV-1/SHV-1 Week 6
Killed SHV-1	MTT[b]	1.9 ± 0.5	2.7 ± 0.7	2.1 ± 0.4[b]	2.3 ± 0.3[b]
	CL[c]	3.3 ± 1.0	3.3 ± 0.9	3.4 ± 0.9	2.7 ± 0.8
Live SHV-1	MTT	1.6 ± 0.4	2.6 ± 0.6	1.5 ± 0.6[b]	1.8 ± 0.7[b]
	CL	2.2 ± 0.8	2.3 ± 0.8	0.9 ± 0.2[c]	0.8 ± 0.1[c]
None	MTT	2.0 ± 0.6	3.1 ± 0.9	4.5 ± 1.3	4.4 ± 1.3
	CL	3.4 ± 0.9	3.4 ± 1.1	3.4 ± 0.9	3.4 ± 1.0

[a] Based on 2.5 × 10^6 cells/test for pigs (n = 2/group, Table I). Values presented represent 6 replicates/pig according to the above treatment groups. MTT values were computed from the maximum peak absorbance (MPA) of PBMC with and without SHV-1/MPA of PBMC with sodium azide. Chemiluminescence (CL) values were computed from the peak cpm of phagocytic cells with and without SHV-1/MP cpm of phagocytic cells exposed to sodium azide

[b] SI values were significantly lower ($P \leq 0.05$) for cultures from pigs exposed to live-virus followed by in vitro cultivation with either killed SHV-1 or, even lowered more, by introduction of live SHV-1, as shown

[c] SI values were significantly lower ($P \leq 0.05$) for PBMC cultures from pigs exposed to live-virus than for PBMC cultures from pigs of other treatment groups, except with live SHV-1 in control and killed-SHV-1 vaccinated groups

Antigens, Inc., Memphis, Tennessee), and a gpX-SHV-1 HerdChek enzyme-linked immunosorbent assay (ELISA; Agritech, Portland, Maine).

SPLEEN CELLS

Spleens were removed from the pigs at necropsy and minced with scissors into cell suspensions before culturing in RS-medium. Macrophages incubated with carbonyl iron were removed from nonadherent spleen cells by use of a magnet (see above).

CHEMILUMINESCENCE ASSAY

The chemiluminescence (CL) response of porcine blood phagocytic cells was measured using a blood cell isolation kit procedure (Los Alamos Diagnostics, Los Alamos, California). Phagocytic cells were assessed for CL while incubating with either opsonized SHV-1 and/or zymosan A containing luminol (5-amino-2, 3-dihyro-1,4-phthalazinedione) (13). The assay was performed in polypropylene vials using a liquid scintillation counter set on an out-of-coincidence mode to obtain background values. Zymosan-opsonized normal porcine serum was prepared from freshly pooled swine serum that had been incubated with zymozan A (1.5 mg/mL) for 20 min at 37°C. After incubation, the zymozan particles were removed by centrifugation and the opsonized serum was stored at −70°C. For measuring extracellularly generated CL, phagocytic cells were incubated with medium containing 1 mM sodium azide. SI values were computed as shown in Table III.

TETRAZOLIUM-BASED COLORIMETRIC ASSAY

A rapid 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, Missouri) colorimetric assay for cell growth and survival was modified and used as a PBMC assay (14). The assay was performed in microtiter plates. The PBMC (about 1 × 10^5 cells/mL) were incubated with and without SHV-1 for 48 h. Cells were then incubated an additional 4 h at 37°C with MTT. The MTT is reduced to purple formazan by mitochondrial dehydrogenases present in intact metabolically active cells. Formazan was concentrated by centrifugation (1000 × g for 2 min), and dissolved in dimethyl sulfoxide. Absorption of formazan was read at 570 nm in a UV_{max} microtiter plate reader. SI values were computed as shown in Table III.

CELL CYTOTOXICITY (CC) ASSAY

Porcine kidney (PK_{15}) cells were labelled with ^{51}Chromium as previously described (7). SHV-1 (strain P-2208) was inoculated onto confluent monolayers of porcine kidney (PK_{15}) cells in MEM (supplemented with 10% FBS, sodium bicarbonate, sodium pyruvate, lactalbumin hydrolysate, and antibiotics noted in RS-medium) and incubated for 1 h at 37°C. The monolayers were drained of medium, rinsed, and incubated in MEM for 48 h. The cells were trypsinized, washed with medium, and resuspended in MEM. The cells were then incubated with 200 uCi $Na_{2_{51}}CrO_4$ (Amersham, U.K.) for 2 h at 37°C, washed 3 times in MEM, and assessed for radioactivity.

Aliquots (100 uL) of effector cells (5 × 10^5/mL, PBMC or PBMC-depleted of macrophages, were dispensed into wells of microtiter plates. Equal volumes of RS-medium, control (SHV-1-negative serum), or SHV-1-positive serum, and ^{51}Cr-labelled target cell suspensions (5 × 10^3/mL) were then added (effector to target, 100:1). The test was performed in 6 replicate wells per group. Test plates were incubated (as above) for 18 h at 37°C. After incubation the cultures were centrifuged for 10 min at 400 × g. The supernatants (100 uL/sample) from each well were collected. The amount of ^{51}Cr radioactivity for each sample was measured by a gamma counter. The percentage cytotoxicity (specific lysis) was calculated as: % cytotoxicity = (experimental mean cpm minus the mean cpm of spontaneous release (SR))/(mean cpm of maximal release (MR) minus the mean cpm of SR) × 100; where SR is defined as the radioactivity released from target cells incubated in medium alone, and MR as cpm in the supernatants of target cells lysed with Triton × 100 (Table IV).

STATISTICS

Statistical significance was determined by using analysis of variance and the least significance difference test for multiple group comparisons (15).

RESULTS

TOTAL AND DIFFERENTIAL BLOOD CELL COUNTS IN CONTROL, SHV-1-VACCINATED, AND SHV-1-INFECTED PIGS

Blood samples from the SHV-1 seronegative pigs bled for 4 (−3 to 0) consecutive wk revealed a mean

TABLE IV. ^{51}Cr-release assay responses of porcine PBMC and spleen cells from control-, killed SHV-1 vaccinated-, and rSHV-1/virulent SHV-1 exposed- pigs[a]

Culture Additive	Treatment Groups of Pigs Mean percent ^{51}Cr (± SD) released from target cells					
	[seronegative]	[vaccinated]		[exposed to live virus]		
		Killed SHV-1		rSHV-1-	rSHV-1/SHV-1	
	Control Week 0 ($n = 2$)	Week 4 ($n = 2$)	Week 6 ($n = 2$)	Week 4 ($n = 2$)	Week 6 ($n = 2$)	Week 10 ($n = 2$)
PBMC, +Ab[a]	8 ± 1.8	20 ± 9.1	18 ± 2.6	25 ± 7.8	5 ± 0.6[b]	20 ± 3.1
PBMC, −Ab	2 ± 0.4	15 ± 4.2	12 ± 4.0	11 ± 3.2	28 ± 5.0	15 ± 4.0
PBMC minus adherent[b] cells, +Ab	5 ± 0.7	15 ± 4.1	12 ± 3.6	13 ± 4.0	4 ± 1.4[b]	35 ± 8.7
PBMC minus adherent cells, −Ab	1 ± 0.4	13 ± 3.8	11 ± 3.2	10 ± 3.8	26 ± 7.1	17 ± 6.8
Spleen, +Ab	9 ± 2.3	ND[c]	15 ± 2.2	ND	ND	16 ± 1.7
Spleen, −Ab	5 ± 1.0	ND	5 ± 0.9[d]	ND	ND	7 ± 1.0[d]
Spleen minus adherent cells, +Ab	8 ± 2.8	ND	25 ± 6.5	ND	ND	19 ± 6.1
Spleen minus adherent cells, −Ab	7 ± 3.1	ND	8 ± 3.6[c]	ND	ND	7 ± 2.9[d]

[a] Autologous serum was used as a source of SHV-1 antibody (+Ab, 1:64 VN titer); −Ab, SHV-1 seronegative serum was used. Adherent cells were removed by use of carbonyl iron engulfed and retrieved by magnet and by plastic adherence

[b] Values were significantly ($P \leq 0.05$) lower for these PBMC cultures from virus-exposed pigs than for comparable PBMC cultures from vaccinated-, rSHV-1-, and rSHV-1/SHV-1 (week 10) pigs

[c] ND, not done

[d] A significantly lower ($P \leq 0.05$) amount of radioactivity was detected in these cultures from pigs challenged with virulent SHV-1 without Ab verses comparable cultures administered Ab

leukocyte cell count ± SD/mL of 125 ± 12 × 10^5 (Table I). These cells were distributed as follows: neutrophils (bands) 5.5 ± 1.7%; neutrophils (segmented) 27 ± 6.5%; eosinophils 4.2 ± 1.9%; basophils 2.9 ± 0.9%; lymphocytes 55.7 ± 4.5%; and monocytes 1.8 ± 0.8%. There were no significant changes in total white blood cell (WBC) and polymorphonucleated cell (PMN) counts between the pigs. Percentages of T- and B-lymphocytes ranged, respectively, from 59 to 51% and 31 to 26%. Serum samples of the pigs were verified as SHV-1 seronegative by the VN and LA assays.

Pigs given the killed-virus vaccine showed comparable WBC and PMN numbers, and percentages of B-lymphocytes to values observed for control pigs. Percentages of T-lymphocytes from vaccinated animals and from control animals ranged in relative ratios from 0.90 to 0.83. When 4 of the killed-virus vaccinated pigs were challenged with virulent SHV-1, WBC, and PMN numbers remained relatively constant in the 5th and 6th wk intervals. However, the percentages of T- and B-cells at the 2 wk postchallenged (PC) interval were significantly ($P \leq 0.05$) lowered.

Pigs receiving live-avirulent rSHV-1 vaccine showed little change in either WBC, PMN counts, or percentages of B-lymphocytes over control animal bleedings. Percentages of T-lymphocytes were affected at the 2nd and 3rd wk PC. When these pigs were challenged with live virulent virus they showed a significant ($P \leq 0.05$) drop in percentages of both T- and B-lymphocytes at 2 wk PC, resulting in respective relative ratio changes of 0.59 and 0.72. By wk 7 and 8, T- and B-cell percentages increased. Following a rechallenging of 2 pigs at the 8th wk, WBC and PMN counts and percentages of T- and B-cells remained lower at the 9th and 10th wk than observed for values observed earlier for control pigs. The SHV-1 rechallenged pigs developed some clinical signs indicative of SHV-1 infection, i.e., hyperthermia (≥40.1°C), and inappetance. They recovered within 7 d PC.

SERUM ANTIBODY RESPONSES AND ISOLATION OF SHV-1 FROM SHV-1-VACCINATED OR SHV-1-INFECTED PIGS

The geometric mean VN serum antibody titer ($\log_2$) of pigs vaccinated IM with killed SHV-1 ranged from 0.2 to 4.5 at the 3rd and 4th wk postvaccination. Pigs exposed IN with rSHV-1 showed similar responses during the same PC period. Following exposure to virulent SHV-1, their VN antibody responses ranged from 3.5 to 6.5 in the 3rd wk PC period. Sera from the pigs, 1 to 4 wk postvaccination, showed positive SHV-1 antibody titers of ≥4 of the VN test. In the rSHV-1 vaccinated pigs, the HerdChek ELISA test detected presence of antibody to glycoprotein X of rSHV-1, at the 2nd to 4th wk postexposure, with optical density ratios (sample: negative) of ≤0.6.

SHV-1 was not isolated from PBMC cultures of weekly blood samples from any of the seronegative- and killed SHV-1-vaccinated pigs that were SHV-1 challenged (Table I). Also, SHV-1 was not isolated from PBMC cultures of weekly blood samples from pigs exposed IN to rSHV-1. When these pigs were IN challenged with virulent SHV-1, rSHV-1 was isolated from PBMC cultures of 2 of the 4 pigs at PC weeks 5, 6, 7, and 8. The 2 positive pigs showed PBMC with SHV-1 beta-galactosidase activity, typical of rSHV-1. Based on microscopic fluorescent antibody (FA) observations and isolation of the virus from PBMC, a very low PBL SHV-1 infectivity of ≤0.0002% was estimated for the pigs. No virus was isolated from PBMC cultures of the pigs at the 9th and 10th wk.

PROLIFERATIVE RESPONSES OF PBMC FROM SHV-1-VACCINATED- OR SHV-1-INFECTED-PIGS.

Since T-lymphocytes of pigs challenged with virulent SHV-1 were affected at the 2nd and 3rd wk PC, and showed a significant ($P \leq 0.05$) drop in numbers following rechallenge, a composite comparison of PBMC cultures from the control and experimental animals was made as shown in Table II. To stimulate proliferative responses, the PBMC cultures (with and without an inoculum of SHV-1) were incubated with the T-cell stimulator, ConA, in the presence and absence of cytokines. SI values for PBMC cultures without cytokines from the seronegative (control) and rSHV-1/SHV-1 animals were not significantly lower than PBMC from killed virus vaccinated-animals. SI values for PBMC from the control animals showed significantly ($P \leq 0.05$) lower values for cultures

incubated with IL-1 and the combination of IL-1/IL-2 than observed for comparable cultures from killed virus vaccinated animals. SI values for PBMC cultures from control and killed virus vaccinated animals incubated with either IL-1/IL-2, IL-1/IL-2/LSP, IL-2, or IL-2/LSP were significantly ($P \leq 0.05$) higher than for comparable PBMC cultures from pigs of the live virus rSHV-1/SHV-1 group at wk 6. Values for SHV-1-infected PBMC cultures from control and killed virus vaccinated-animals incubated with LSP were significantly ($P \leq 0.05$) lower than for comparable PBMC cultures incubated with either IL-1, IL-1/IL-2, IL-2, IL-2/LSP, or IL-1/IL-2/LSP. When LSP was incubated with PBMC cultures from rSHV-1/SHV-1 pigs, there was no enhancement of proliferative responses of the cultures either with or without an inoculum of SHV-1. PBMC cultures from the rSHV-1/SHV-1 pigs were generally affected by introduction of an inoculum of SHV-1, resulting in lowered proliferative responses.

Following exposure of PBMC cultures from control, killed virus vaccinated-, and live virus exposed- pigs to SHV-1, it was observed that the virus affected mitochondrial and phagocytic activities of PBMC as shown in Table III. When MTT and CL values were obtained from PBMC control cultures, and compared to cultures exposed to either killed SHV-1 or live SHV-1 from control and killed virus pigs, there appeared to be little differences between results of the control and experimental animals. When PBMC from pigs exposed to either rSHV-1 or rSHV/SHV-1 were incubated with either killed or live virus and analyzed by the MTT test, SI values were significantly ($P \leq 0.05$) lower than for comparable PBMC cultures without killed or live virus. Similarly, when PBMC cultures from pigs exposed to rSHV-1/SHV-1 were inoculated with live SHV-1 and compared to comparable PBMC cultures exposed to killed virus or no virus (none), they showed significantly ($P \leq 0.05$) lowered CL SI values. In these studies, sodium azide effectively suppressed mitochondrial dehydrogenase and CL activities of PBMC. Macrophages were proficient in responding to opsonized zymozan in the CL assay.

In Table IV are the results depicting CC activity of PBMC from control-, killed virus vaccinated-, and live virus exposed- pigs to SHV-1. From the data, PBMC and spleen cells from control-, killed virus, and rSHV-1/SHV-1- (10 wk) pigs showed higher CC activity in the presence of SHV-1 antibody than in its absence. These results indicate that SHV-1 antibody augments CC activity. However, when PBMC (with and without adherent cells) from rSHV/SHV-1 pigs were incubated with SHV-1 antibody and tested for CC responses, the results were variable, being significantly ($P \leq 0.05$) lower at wk 6, and higher at wk 10. From the PBMC cultures, rSHV-1 was isolated at wk 6 but not at wk 10. When PBMC cultures were incubated without adherent cells, but with SHV-1 antibody at the wk 6 and 10 intervals, CC responses were low at wk 6 and very high at wk 10. No attempt was made to isolate virus from the PBMC cultures without adherent cells. Spleen cells, with and without SHV-1 antibody, showed similar CC responses to comparable PBMC cultures. Addition of SHV-1 antibody to spleen cells from killed virus-challenged (wk 6)- and rSHV-1/SHV-1 (wk 10)-pigs showed significantly ($P \leq 0.05$) lower CC responses than for comparable spleen cells with antibody. No virus was isolated from any of the spleen cell cultures.

DISCUSSION

Understanding the nature of SHV-1-host interactions will allow development of rational approaches to management of infected pigs. This is made difficult, however, by the fact that SHV-1, like other herpesviruses, may affect B-cells, causing a specific suppression of Ig, and also affect T-suppressor/cytotoxic cells (8, 16–18). To define the various cell-mediated interactions of host defense mechanisms and SHV-1 components that result in clinical disease, in viral persistence, or in the control of the effects of viral pathogenesis is a major challenge.

The observation of infectious virus in close association with circulating PBMC supports the hypothesis that SHV-1 infected immune cells may provide an alternative mechanism for trafficking of virus throughout the body of the host animal, particularly to sites in which neural spread is unlikely (2–4,19–22). Virus was only detectable at low frequency following stimulation of PBMC with ConA, suggesting T-cell stimulation, and IL-2 stimulation of protein kinase C with PBL plasma membranes (23). The requirement of prolonged incubation on PK_{15} cells through cocultivation before appearance of CPE may reflect delayed virus reactivation or a requirement of PBL lysis for release of intracellular virus as previously suggested (21). Recently, it was found that a herpesvirus isolated from human CD4+ cells could only be isolated from a healthy subject following incubation of cells under conditions promoting T-cell activation (24). Others have shown (25) that the clearance of herpes simplex virus 1 from infected mice could be enhanced by administering antigen-sensitized lymphocytes that were collected from syngeneic mice and exposed to IL-2 in vitro. Additionally, toxin from *Pasteurella multocida* serogroup D has been reported to enhance SHV-1 replication in vitro and increase mortality of pigs infected with a sublethal dose of SHV-1 (26). This indicates the synergy effects of bacteria-virus infection on aggravating the outcome of SHV-1 infection.

The addition of ConA, IL-2 and gliotoxin leading to enhanced SHV-1 replication is not known, but is likely to involve cellular functions that are associated with cell differentiation. In our isolation procedures, the mitogen and cytokine would favor T-cell activation. With gliotoxin, we had earlier shown it to be antiphagocytic and a stimulator of PBL proliferation (Williams, unpublished data). Co-cultivation on PK_{15} cells appears suitable as a cell line isolating the virus (27). Prolonged periods of culture were necessary for detecting virus that may be intracellular in PBL (21). It appears that PBMC are less permissive to SHV-1 infection than porcine alveolar macrophages (5). The role of nonessential glycoproteins (gp) gI, gp63, and gIII in the release of SHV-1 from different cell lines have been clearly shown to play a role in modulating viral replication (28). The differential effects of the gp on the

release of virus from different types of cells may be related to the ability of the virus to replicate, spread, and destroy different target cells. In principle these gp may therefore contribute significantly to virulence and to the pathogenesis of the virus.

The mechanism of the decrease in CC activity following SHV-1 infection is not known. The observed low CC activity in PBMC from challenged pigs, at wk 6 Pc, might be the result of a decrease in number of killer cells or a decrease in lytic potential. The adherent population, which constitutes 5 to 10% of total PBMC, has been reported to support virus replication after in vitro infection, whereas nonadherent cells do not (29). Removal of phagocytic cells from PBMC caused an increased CC activity; such a treatment was inefficient with spleen cell preparations, due to a very low proportion of phagocytic cells in the original spleen preparations. No SHV-1 was detected in spleen samples of any of the challenged and vaccinated and challenged pigs that were necropsied on week 10. However, SHV-1 was isolated from PBMC cultures of challenged pigs. The decrease in activity in control and infected PBMC, compared with initial cultures, could be caused by reduced function of effector cells or prolonged incubation, or to the presence of excess IgG affected B-lymphocyte activities (30). Infection of total PBMC cultures in our experiments did not result in an increase in virus yield or in reduction in CC activity, compared with those in noninfected controls. Loss of cell viability does not appear to be a major factor, although recently it was reported that SHV-1-infected mixed breed pigs developed a moderate leukopenia. There was a loss of up to 40% of monocytes and up to 50% of lymphocytes (21). The lymphopenia resulted from reduction in both circulating CD4+ and CD8+ lymphocytes. We have recently reported that rSHV-1 in in vitro studies has been shown to affect T-lymphocytes causing an unresponsiveness to ConA despite proper expression of IL-2 receptors (22).

The nature of the activation signals generated by mitogens on T-cell receptor sites is poorly understood. It is known that T-cells can be activated in vivo and in vitro by antigen and/or mitogen to secrete and express specific membrane receptors for IL-2. The subsequent interaction of IL-2 with its receptor stimulates the T-cell to proliferate (8,31). In the present study, we found that the presence of exogenous human interleukins IL-1, IL-2, and LSP, alone or in combinations could not reverse the mitogen-stimulated PBL immunosuppression by SHV-1. In other reports, IL-2 has been shown to reverse suppression induced by a 5-lipoxygenase inhibitor (32). Exogenous IL-2 has been demonstrated to partially restore the in vitro lymphocyte proliferation in response to phytohemagglutinin (PHA) from lymphadenopathy patients (33). Also, human rIL-2 administered to pigs, vaccinated with an SHV-1 subunit vaccine, enhances their immunological responses to SHV-1 (34). Recently we have demonstrated that PBL of neonatal piglets, immediately after birth, strongly respond to PHA and ConA, both potent mitogens for T-lymphocytes of adult swine as well as to pokeweed mitogen which has been used as a T-cell dependent B-cell activator (29,35).

It is known that IL-1 can act as a co-stimulator for T-cells by enhancing the transcription and secretion of the autocrine T-cell growth factor, IL-2 (36) and by stimulating the expression of membrane receptors for IL-2 (37). Thus, the combination of enhanced secretion of growth factor and receptor expression can account for the T-cell function of IL-1. It has been reported human rIL-1 can stimulate production of diacylglycerol (DAG) and phosphorylcholine from phosphotidylcholine in the absence of phosphatidylinositol turnover in a human T-cell line (38). In turn, DAG stimulates the production of protein kinase C (PKC), which plays an important role in IL-2 production and in IL-2 receptor development from PHA-stimulated human T-cells (39). Others have demonstrated that IL-2 stimulates elevation in phosphotidylinositol metabolism and PKC translocation to membranes (23). The enzymes PKC and protein tyrosine kinase (PTK) play important roles in human T-cell proliferation, and IL-2 production (39). It is not known whether SHV-1 can affect either the translocation of the cytosolic PKC to the membrane or block the receptor-associated gene with PTK enzyme.

The finding of only rSHV-1 was surprising in that no strain P-2208 was found. This may indicate that only the recombinant virus can survive in the host or that transfections occur resulting in recombinants dissimilar from either rSHV-1 or SHV-1. Recently, we have found that a Minnesota miniature sow vaccinated with rSHV-1 and challenged with SHV-1, prior to gestation, at farrowing delivered 5 piglets. Three of these piglets died after delivery, and 2 survived. Both of the surviving pigs SHV-1 seroconverted, with one of them becoming blind. Both pigs became persistently infected with SHV-1, and virus could be periodically isolated from their PBMC. Analysis of the SHV-1 indicated that the viral isolates were beta-galactosidase positive, indicating the presence of recombinants.

The identification of SHV-1-vaccinated pigs that have been infected with field strains of SHV-1 is of paramount importance in controlling the spread of Aujeszky's disease. The current approach to vaccination centers on the use of avirulent deletion mutant viruses whereby genes encoding one or more viral proteins are deleted using recombinant DNA methodology. Although deletion mutant vaccines have been shown to be efficacious, recent studies have shown that in vivo recombination between modified-live deletion mutant vaccines and virulent challenge virus does occur (40). As a result there is a potential for the emergence of virulent marker negative strains of SHV-1 in the field. If the risk of in vivo recombination of modified-live deletion mutant vaccines with virulent field virus is perceived to pose a threat to national SHV-1 eradication efforts, this approach will need to be further evaluated and modified. Also, the antigenic cross-relationship of SHV-1 to porcine cytomegalovirus is not known and is an area that needs to be clarified (17).

Recent reports on assessing SLA expression after virus inoculation have implicated modulation of expression of SLA class I and class II antigens during infection as a likely

influence on humoral and cellular antiviral immune responses (41,42). These studies indicate that, as we better understand the genetic structure of the SLA complex in inbred miniature pigs, we should be able to define exactly the SLA encoded genes and their function in regulating vaccine and disease responses.

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