# Canine parvovirus vaccine elicits protection from the inflammatory and clinical consequences of the disease

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*Inflammatory changes following infection are central to the clinical manifestation of disease. However, information regarding such changes in animal disease is limited. In canine parvovirus infected puppies we measured the levels of acute phase proteins and changes in leukocyte phenotypes and cell trafficking by flow cytometry. These parameters correlated with conventional assessment of clinical disease in a vaccine efficacy study. Seropositive (CPV-2) 6-week-old puppies given three doses of a CPV-2 containing vaccine developed significant antibody titers and remained healthy after experimental infection with CPV-2b. Unvaccinated controls developed clinical signs and shed virus. Importantly, acute phase proteins became elevated, and lymphopenia, neutropenia and modulation of neutrophil-CD4 were detected in controls but not in vaccinates. © 1997 Elsevier Science Ltd.*

Canine parvovirus type 2 (CPV-2) was described as a new canine pathogen in 1978[1]; however, the new strains CPV-2a[2,3] and CPV-2b[4] quickly emerged. Today CPV-2a is most common in Europe while CPV-2b represents *ca* 80% of current field isolates in the United States[5]. In puppies this virus replicates in mitotic cells[6,7] and is transmitted by the fecal–oral route causing a fulminating enteric disease. Initial infection most likely occurs in the tonsil, however, the virus rapidly spreads to other lymphoid tissues and to crypt cells in the gut[8]. Crypt cell death and erosion of the enteric lining results in the vomiting and diarrhea characteristic of this disease. Protection in very young puppies is mainly by passive transfer of maternal antibody to offspring. However, as maternal antibody declines, puppies become highly susceptible to infection. Susceptibility to parvovirus infection often coincides with the time that puppies are separated from the dam, significantly increasing their risk of exposure[9].

Clinical signs resulting from experimental challenge with the CPV-2 strain are typically mild[10], and not representative of the virulent disease observed after natural infection with the CPV-2b strain that is more prevalent in the United States[11]. Lymphopenia, usually evaluated by a manual differential cell count can be highly variable, and neutropenia, commonly described in natural cases of infection[12], is inconsistently observed after experimental challenge[7]. Nevertheless, evaluation

of these parameters and reduction of viral shed have proven useful in assessing vaccine efficacy.

Our understanding and ability to evaluate clinical disease constantly changes as technologies and reagents are developed. For example, flow cytometry is being utilized more frequently in diagnostic situations because cell specific, quantitative data regarding cell populations can be obtained[13]. When combined with an automated total white cell count this information may yield more powerful, less variable analysis of cell surface proteins and leukocyte trafficking. Similarly, the acute phase response is well known as a nonspecific response to pathogenic injury[14]. Despite the nonspecific nature of this response, recent evidence has shown that the stimulus for production of acute phase proteins by the liver is a tightly regulated response to cytokines such as interleukin-1 (IL-1) and IL-6 which are released soon after infection[15-17]. In addition, the production of specific antibody reagents has fostered the development of quantitative assays increasing the accuracy and specificity of detecting acute phase reactants. Thus, elevated levels of one or more of these pro-inflammatory proteins may be predictive of clinical infection and disease. Elevated levels of IL-6, $a$-1 acid glycoprotein ($a$-1AG), and serum amyloid A (SAA) have been described in association with acute as well as chronic diseases[18-20]. The impact of vaccination on these parameters after infectious challenge has not been examined.

In this study we evaluated whether measuring levels of acute phase proteins and investigating changes in leukocyte phenotypes by flow cytometry would complement conventional clinical assessment of a vaccine efficacy study. The association of these parameters with the

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major clinical signs of parvovirus induced disease in vaccinated vs nonvaccinated animals is described for seropositive puppies given a CPV-2 vaccine followed by experimental infection with CPV-2b. Specific emphasis was placed on measuring CPV specific neutralizing antibodies and two acute phase reactants, a-1AG and SAA. Changes associated with leukopenia were characterized using flow cytometric analysis.

## MATERIALS AND METHODS

### Animals

Six-week-old mongrel puppies (Alder Ridge Farms, BWEF Inc., Lakewood, PA) with maternally derived CPV-2a serum neutralizing (SN) titers ranging from <2–1024 were used. Fifty puppies were randomized into two treatment groups ($n=25$) based on sex, litter, and date of birth; each group had a similar range of SN titers.

### Vaccine

Puppies were vaccinated subcutaneously at 6, 9, and 12 weeks of age with an experimental CPV-2 parvovirus modified-live vaccine having a titer >6 logs $TCID_{50}$ $ml^{-1}$. The parvovirus component was delivered in combination with distemper-adenovirus type 2-parainfluenza, killed corona virus, and *Leptospira canicola-icterohaemorrhagia* bacterin (Pfizer, Inc., Lincoln, NE). A placebo consisting of Earles Balanced Salt Solution (BioWhittaker, Walkersville, MD) was given subcutaneously to the control group in order to simulate the status of nonvaccinated puppies in the field.

### Serum neutralization titers

Serum samples were collected prior to the first vaccination and then at 1–3 week intervals for antibody titers. Serial twofold dilutions of heat inactivated serum samples were made in 96-well microtiter plates. Samples were incubated with CPV-2a (50–300 $TCID_{50}$) virus for 90 min at 37°C. Dog kidney cells (ATCC-CCL34) ($6 \times 10^5$ per well) were added and incubated for three more days at 37°C. Each plate contained a virus and cell control. To visualize virus infected cells, the monolayers were fixed with 80% acetone, then reacted with a monoclonal antibody (mab) (1:10000) specific for the major capsid protein (VP1), followed by fluorescein conjugated anti-murine IgG (Kierkegaard and Perry International, Gaithersburg, MD). Wells were considered positive for virus if three or more than three cells in a focus were fluorescent. The endpoint neutralization titer was determined as the reciprocal of the final serum dilution where one or less than one of two wells had less than three positive cells.

### Challenge virus

The CPV-2b was obtained from the National Veterinary Services Laboratory, Ames, IA. The challenge dose for this study was determined previously[21] and given orally and intranasally ($10^{3.2}$ $TCID_{50}$ per dose) at 15 weeks of age.

### Clinical observations

Clinical scores were based on *clinical attitude* [normal=1, anorexia=2, depression=5, moribund

(euthanasia)=15, death (between observations)=20]; *vomiting* (food only=2, mucus and food=3, mucus only=4); *dehydration* (slight=1, severe=2); and *quality of feces* (formed=1, nonformed=2, formed with mucus=3, formed with blood=4, formed with blood and mucus=5, nonformed with mucus=6, nonformed with blood=7, nonformed with blood and mucus=8). Significant clinical scores were calculated as the mean plus 2S.D. of day 0 scores for all 50 puppies. Puppies were observed for 14 days post-challenge, however, clinical signs and rectal body temperatures were scored only during the first 10 days. Moribund animals were humanely euthanized during the observation period by the attending veterinarian.

### Virus isolation from fecal samples

Fecal samples were collected daily for 10 days post-challenge and stored at −20°C. To isolate replicating virus the samples were prepared as clarified, 20% (w/v) fecal suspensions in tissue culture medium. Duplicate, tenfold dilutions of the suspension were mixed with feline kidney cells (ATCC-CCL94) ($1.4 \times 10^4$ cells per well) in 96-well microtiter plates and incubated for 3–5 days at 37°C. Cell monolayers were fixed and stained with major capsid protein specific mabs as described above. Virus titer was calculated using a Spearman–Karber equation[22]. $Log_{10}$ titers $\leq 2.2$ $TCID_{50}$ $ml^{-1}$ were below the level of sensitivity of this assay, due to fecal sample toxicity, and scored as no virus shed.

### Flow cytometric and conventional evaluation of peripheral blood leukocytes

Peripheral blood leukocytes (PBL) in EDTA were collected on days −2, 0, 3, 5, 7, 9 post-challenge. Each sample was divided into two aliquots; one was submitted to a commercial laboratory (Roche Diagnostics, Burlington, NC) for total leukocyte count and cell differential analysis, and the other was processed for flow cytometry. PBL were washed free of serum with flow buffer; Hank's Balanced Salt Solution (BioWhittaker) without Phenol Red, $Mg^{2+}$ or $Ca^{2+}$, and containing 0.1% sodium azide, and 0.5% BSA (Fraction V, Sigma Chemical Co, St. Louis, MO). PBL were resuspended to 75% of the original volume in flow buffer. Cells were reacted with optimal dilutions of unconjugated murine mabs specific for canine leukocytes[23,24]. Antibodies specific for the following canine leukocyte subsets were used: CD4+ T-cells (LSM12.125); CD8+ T-cells (LSM1.140); pan T-cells (LSM8.358); B-cells (LSM11.425)[25]; and GM-1+ neutrophils/monocytes (DH59B) (VMRD, Pullman, WA). Specific binding was detected using fluorescein (FITC) conjugated goat F(ab)'2 anti-murine IgG and IgM antibody (Biosource International, Camarillo, CA) previously adsorbed with canine spleen acetone powder (Sigma). Control wells contained unstained cells or cells reacted with the FITC antibody alone. Erythrocytes were removed with FACS® lysing solution (Becton-Dickinson, Mountain View, CA) and cells were fixed using 1% p-formaldehyde in flow buffer and stored at 4°C. Data was acquired on 15000 ungated cells using a FACSTAR Plus (Becton-Dickinson) flow cytometer and analyzed using the PC LYSYS® (Becton-Dickinson) software.
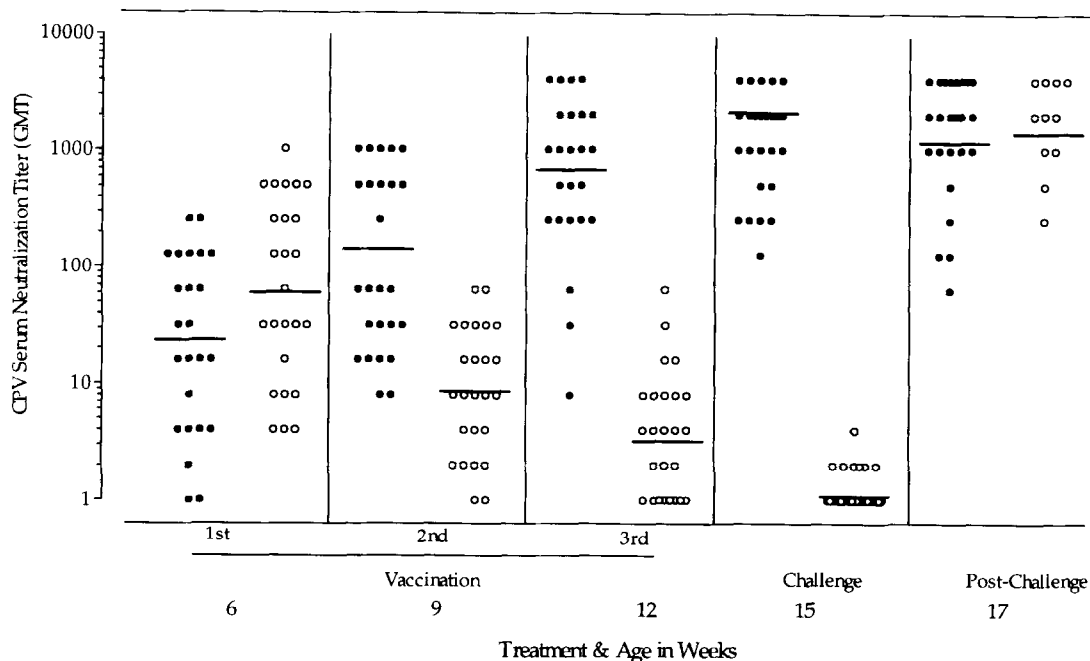
**Figure 1** CPV-2a serum neutralization titers in 6-week-old canine parvovirus seropositive puppies given a three dose vaccine regimen (●) or placebo (○) followed by CPV-2b challenge. The geometric mean titer (GMT) for the group at each time point is indicated by a solid line

Logical gates were drawn around lymphocyte, monocyte, and neutrophil populations based on forward and side light scatter profiles. Then using side scatter vs fluorescence intensity, markers for fluoresence intensity were set based on the negative control. Samples were discounted when erythrocyte lysis was incomplete and significant contamination of the lymphocyte gates prevented accurate analysis. Absolute lymphocyte counts from flow cytometrically analyzed leukocytes were calculated using the total leukocyte count obtained commercially multiplied by the percentages derived from flow cytometric analysis [pan-T cells or (CD4$^+$ plus CD8$^+$ T cells), plus B cells]. These percentages were compared to the cell counts obtained by the manual differential obtained commercially.

### α-1AG

A commercially available canine specific radial immunodiffusion kit (Developmental Technologies, Frederick, MD) was used according to the manufacturer's specifications. Control standards or test serum/ plasma samples (5 μl) were pipetted into precut wells in a gel impregnated with canine α-1AG antibody. After 48 h at room temperature in a humid chamber, the area within the precipitin rings was measured using a BioImage® gel scanner (Millipore Corporation, Bedford, MA). The quantity of α-1AG in test samples was extrapolated from control standards included on each plate.

### SAA

The level of SAA was quantitated by a sandwich ELISA using two mabs with specificity to different epitopes on canine SAA. SAA was captured onto wells of an ELISA plate with one mab and detected with a biotinylated second mab; amplification was strepavidin-horseradish peroxidase. The antibodies and assay were developed in the laboratory of Dr Thomas L.

McDonald, University of Nebraska Medical Center, Omaha, NE. The amount of SAA in an unknown sample was interpolated from a standard curve of known quantities of purified canine SAA. The purity and concentration of SAA in the standard was determined by sequence analysis and amino acid composition because several proteins copurify with canine SAA, rendering standard protein determinations inaccurate. Sensitivity of the assay ranged from 40–2000 pg ml$^{-1}$. Inter and intra experimental variation was <10%.

### Establishment of normal parameters for 15-week-old puppies and statistical tests

Peripheral blood lymphocytes drawn pre-challenge (days − 2, 0) from all 50 puppies were used to establish normal values for all hematological measurements. Upper and lower cutoff values for significance were determined as the group mean ±2 S.D. Differences between groups were evaluated using Fisher's exact test (*P*-values for two-sided tests) or *t*-tests on square root transformed values (*P*-values were based on Cochran and Cox's approximation because of differences in group variances)[26]. Statistical correlations were evaluated by the Spearman Rank method for nonparametric measurements[27].

## RESULTS

### Antibody responses

Seroconversion after vaccination in seropositive puppies was determined as a fourfold or more increase in SN titer from the previous timepoint. Prior to challenge all vaccinated puppies seroconverted, however, this occurred at different timepoints during the three dose vaccine regimen (*Figure 1*). Thirteen puppies seroconverted after the first vaccination (SN titers pre-, <1:2–1:64; post-, 1:64–1:1024). Nine puppies seroconverted after the second vaccination (pre-, 1:8–1:64; post-,

Clinical Scores

Log₁₀ Virus Shed in Feces

A.

B.

Days Post CPV-2 Challenge
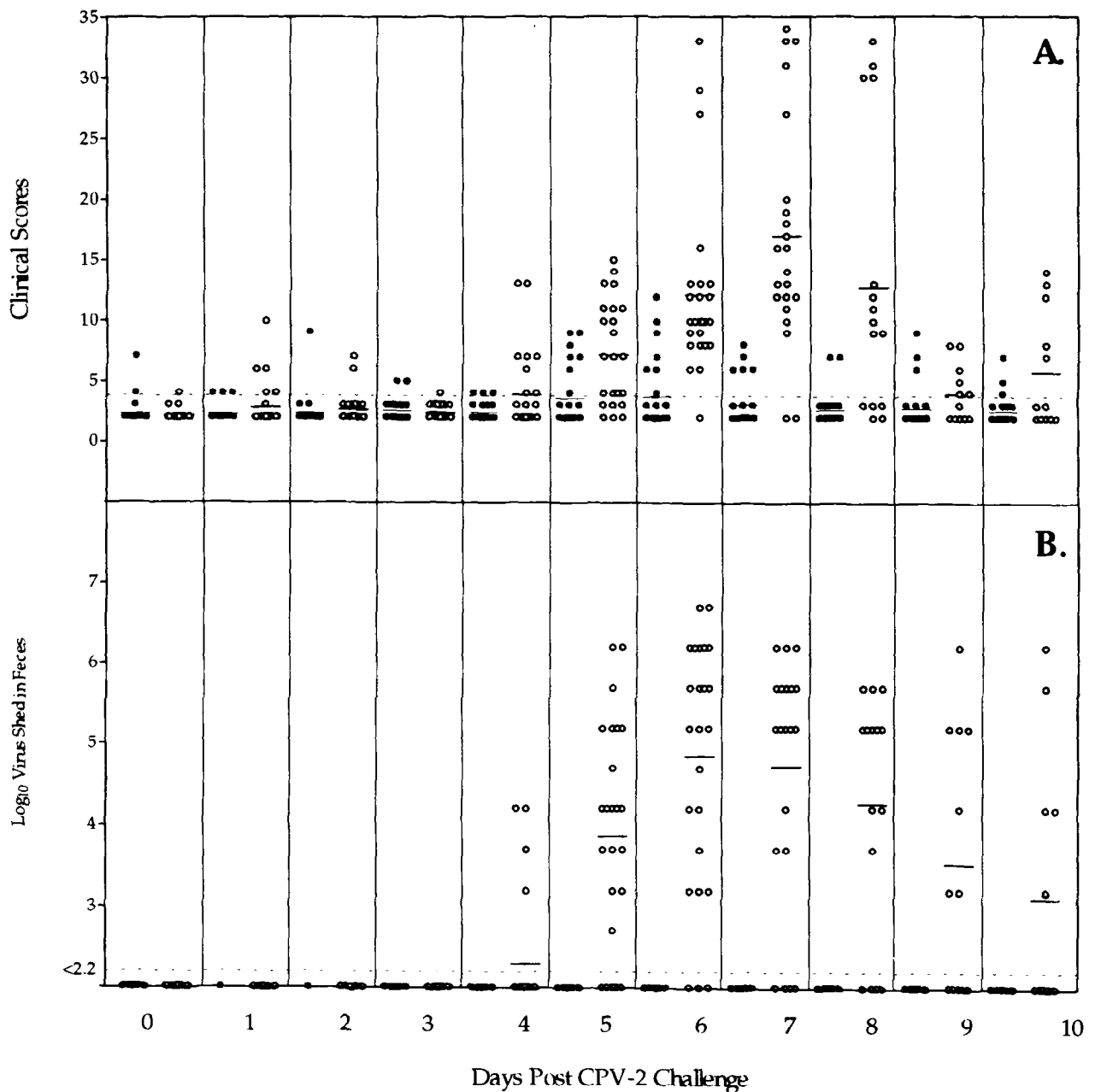
Figure 2 Clinical scores and virus recovery after CPV-2b challenge in individual vaccinated (●) and control puppies (○). Group means are indicated by solid lines. (A) Clinical scores; the upper cutoff for significance from normal for individual animals was determined as the mean of day 0 plus 2 S.D. and is shown as a dotted line. (B) Virus shed; the dotted line indicates the detection limit of the assay

1:256–1:1024). Seroconversion in the three remaining puppies required three vaccinations. Their SN titers prior to first vaccination were 1:128 and continued to drop (range 1:8–1:64 at the time of the second vaccination) before a fourfold increase in titer was observed after the third vaccination. Post-challenge, SN titers in those three puppies were among the lowest (1:64–1:512).

## Clinical response and viral shed after CPV-2b challenge

All 25 puppies in the control group challenged with CPV-2b developed very severe signs consisting of vomiting, diarrhea, depression, anorexia, and dehydration in contrast to mild symptoms typically elicited by a CPV-2 or CPV-2a experimental challenge. The normal mean clinical score on day 0 was 2.24 ± 0.77. Elevated clinical scores were observed beginning day 4 and peaked (mean

12.25 ± 4.91) on day 7 post-challenge (Figure 2A). Fourteen puppies died or were euthanized during the observation period. In contrast, occasional observations of mild gastrointestinal upsets resulted in elevated clinical scores given to some vaccinated puppies. However, these signs were not accompanied by depression, anorexia, dehydration, or virus shed. Fever was not a consistent finding. Elevated temperatures ≥ 39.6°C were observed in 5/25 vaccinates and 8/25 controls on a single day. Only one puppy in the control group experienced three consecutive days with fever.

None of the vaccinated puppies shed detectable levels of replicating virus at any stage of the 10 day observation period (Figure 2B). In contrast, nonvaccinated controls shed virus beginning on day 4, with peak titers at day 6 post-challenge; 5/11 surviving puppies still shed significant levels of virus day 10 post-challenge. There
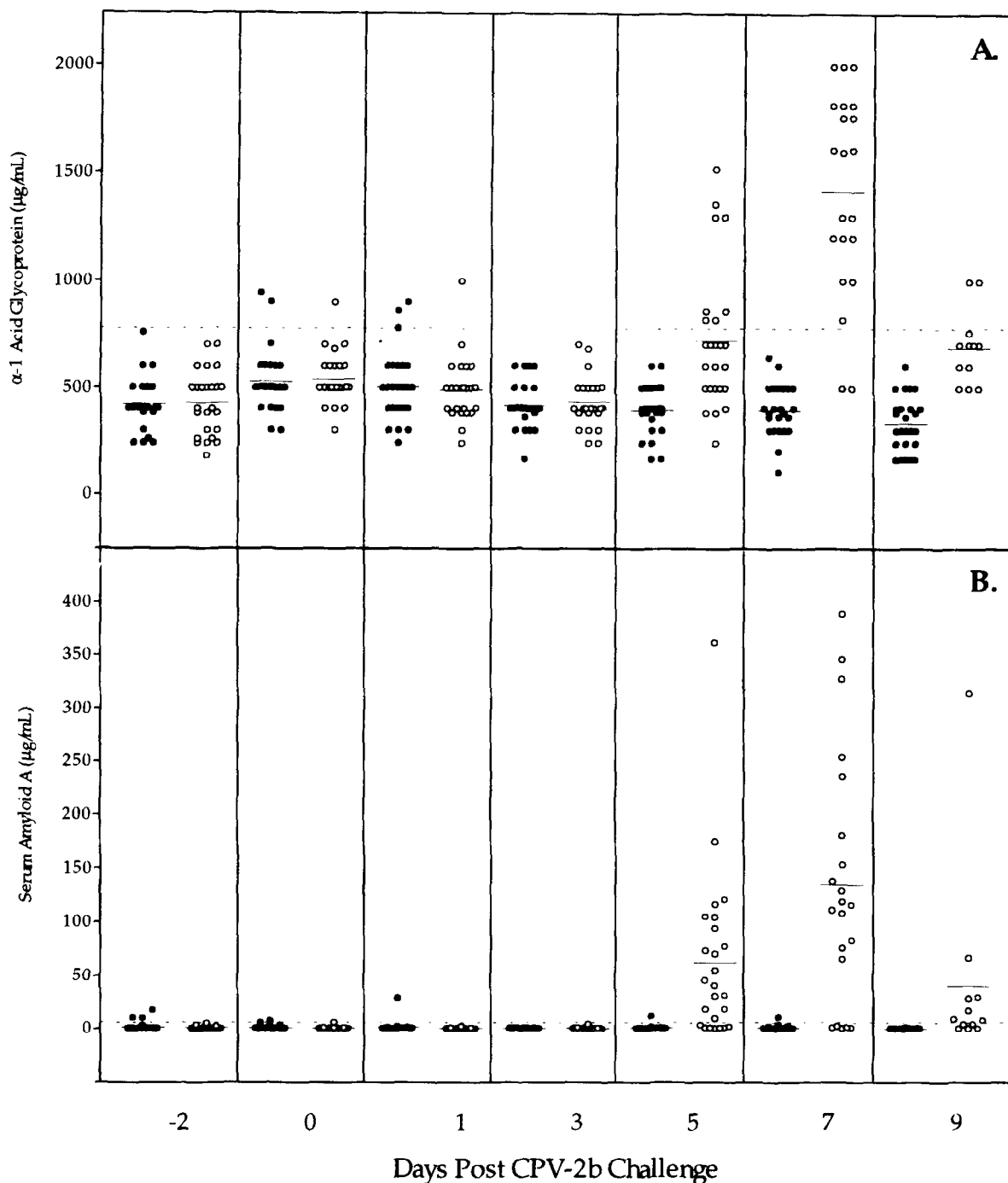
**Figure 3** Acute phase proteins in plasma of 15-week-old vaccinated (●) and control puppies (○) puppies after CPV-2b challenge. Group means are shown by solid line; while the upper limit cutoff for significant deviation from normal (mean plus 2 S.D.) for individual values is indicated by a dotted line. (A) $a$-1AG levels (mg ml$^{-1}$) measured by radial immunodiffusion; (B) SAA levels measured by ELISA

was significant correlation ($P<0.05$) of clinical scores and virus shed on day 5 ($r=0.54$), day 8 ($r=0.64$), and day 9 ($r=0.66$).

**Effect of CPV-2b challenge on acute phase proteins**

Elevated $a$-1AG (two- to fivefold) and SAA (30–800-fold) were observed in 22/25 (*Figure 3A*) and 23/25 (*Figure 3B*) nonvaccinated controls, respectively, beginning day 5 and peaking at day 7 post CPV-2b challenge. Normal mean plasma levels of $a$-1AG and SAA were $480 \pm 149$ $\mu$g ml$^{-1}$ and $1.15 \pm 2.53$ pg ml$^{-1}$, respectively. $a$-1AG levels were elevated in three vaccinates on day 0 or 1 post-challenge. Similarly, SAA levels were

elevated in two vaccinates prior to challenge. Low but significant SAA values were observed in three vaccinates on sporadic days post-challenge, but these values did not coincide with clinical signs, virus shed or hematologic changes. One puppy that died on day 5 did not have elevated levels of either acute phase reactant. Clinical scores significantly correlated ($P<0.05$) with both $a$-1AG ($r=0.54$) and SAA ($r=0.78$) on day 5 and similarly on day 7.

**Effect of CPV-2b challenge on leukocyte trafficking**

Using cell specific antibodies and flow cytometric analysis, a mean of 88% of leukocytes in peripheral

## Days Post CPV-2 Challenge
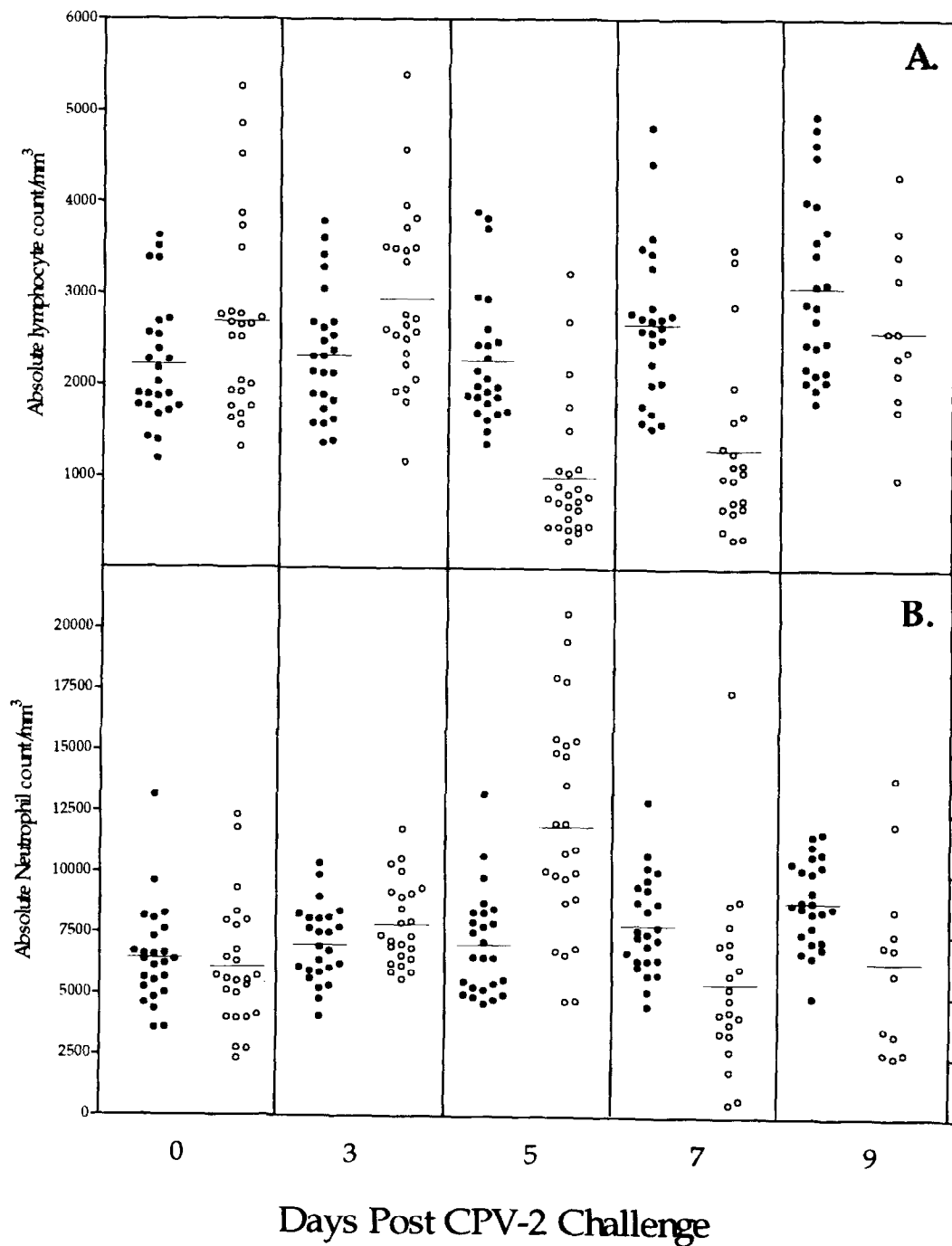
**Figure 4** Absolute lymphocyte (A) and neutrophil (B) counts in peripheral blood of 15-week-old vaccinated (●) and control puppies (○) after CPV-2b challenge. Group means are shown as solid lines at each timepoint

blood were positively identified as T-cells ($CD4^+$ and $CD8^+$, pan-T), B-cells, monocytes, and neutrophils when compared to the absolute leukocyte count obtained conventionally. This is consistent with the fact that antibodies for natural killer cells, basophils, and eosinophils were not available commercially.

Lymphopenia (a ≥50% drop in initial lymphocyte count) was identified in 23/25 puppies in the control group and in 0/25 of the vaccinated puppies, primarily on days 5 and 7 post-challenge (*Figure 4A*) by flow cytometric analysis of peripheral blood. Lymphopenia was due to loss of both T cells and B cells, but was not selective for $CD4^+$ or $CD8^+$ T cells because CD4:CD8 ratios remained constant (data not shown). By compari-

son, using the manual differential cell count, 23/25 control and 4/25 vaccinated puppies were lymphopenic post-challenge (not shown).

Neutrophilia (a ≥50% increase in prechallenge absolute neutrophil counts) was observed in a total of 23/25 nonvaccinated controls between days 3 and 7, and in 12/25 vaccinates between days 5 and 9 post-challenge by both flow cytometric and conventional differential counts. The percent neutrophilia for controls (17/25) was higher than for vaccinates (4/25; $P=0.004$) on day 5. Neutropenia was not observed in any vaccinate during the 10 day observation period. However, in 12/25 (manual differential) compared with 8/25 (flow cytometry) nonvaccinated controls, neutrophilia was followed

**Increasing Fluorescence Intensity**
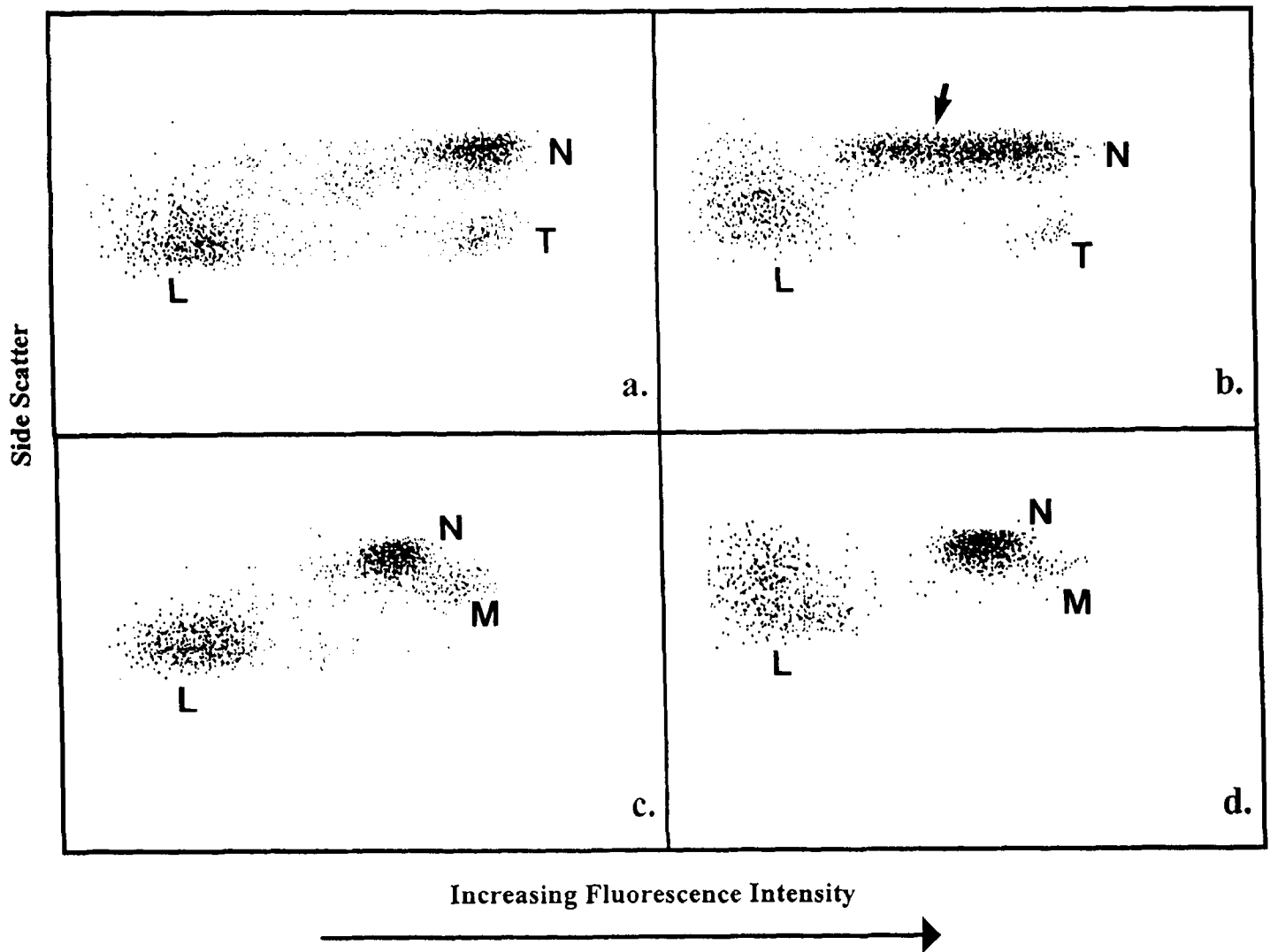
**Figure 5** Comparison of CD4 and GM-1 expression on peripheral blood neutrophils and lymphocytes of a representative nonvaccinated control before (day –2; A, C) and after (day 5; B, D) CPV-2b challenge. (a) Normal CD4 expression on neutrophils (N) and a subset (T) of lymphocytes (L). (b) CD4 expression 5 days after CPV-2b challenge. The arrow points to CD4 downmodulation on neutrophils. (c) Normal GM-1 expression (day –2) on neutrophils (N) and monocytes (M), but not on lymphocytes (L). (d) GM-1 expression 5 days after CPV-2b challenge

by neutropenia on day 7 and was statistically different from vaccinates (0/25; $P$=0.002). Although flow cytometric differential cell counts were less variable on a day to day basis, more timepoints were obtained manually accounting for the discrepancy between the two methods.
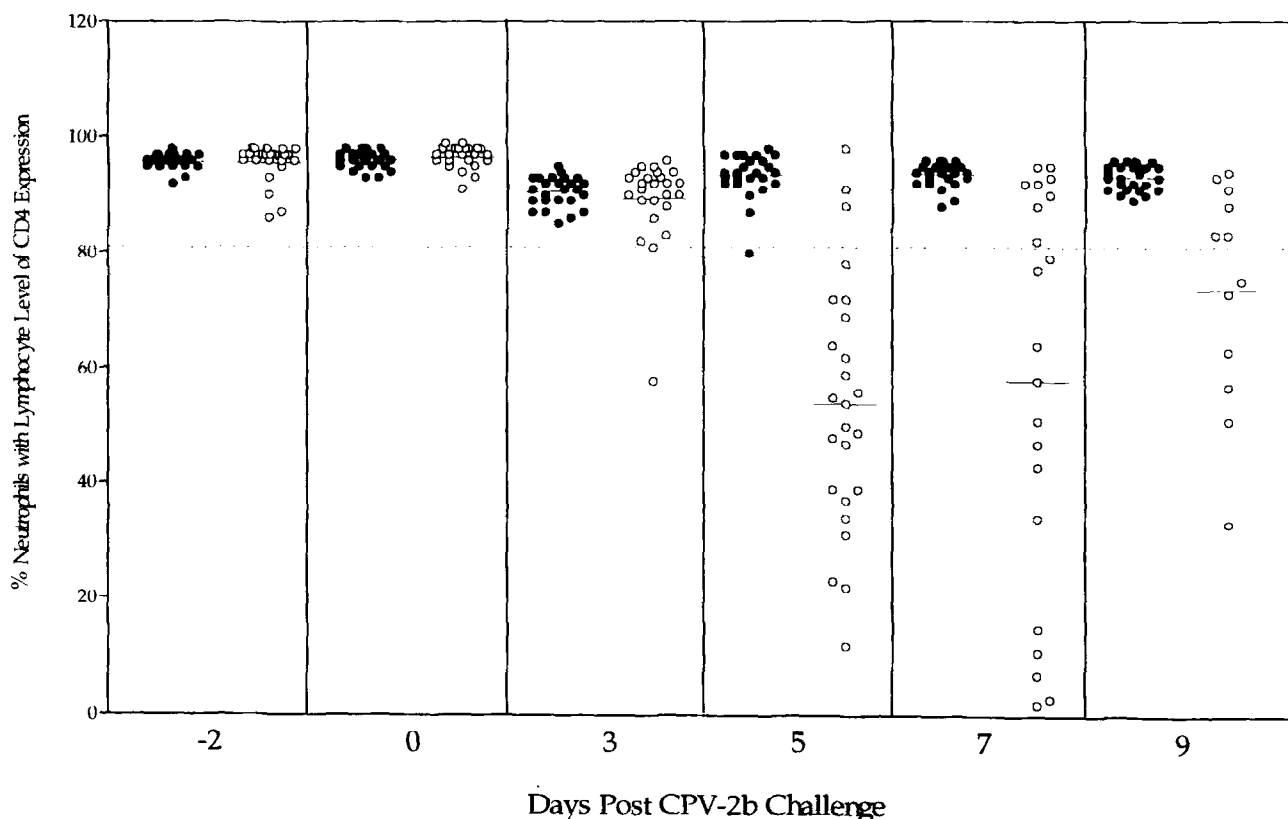
**Effect of CPV-2b challenge on neutrophil-CD4 expression**

Post-challenge, a novel change occurred in the pattern of CD4 expressed on neutrophils when compared to lymphocytes. Normal CD4 expression was equally bright and homogeneous on neutrophils and lymphocytes as determined by the ratio of mean fluorescence intensity of neutrophils to lymphocytes (nonvaccinates=1.17±0.22; vaccinates=1.13±0.24), and is illustrated in *Figure 5a*. However, beginning 3 days post-challenge, the intensity of CD4 expression on neutrophils gradually decreased over a 2–3 day period changing from a bright homogeneous pattern to a smear of cells ranging from dim to bright, and in some animals became completely negative. This change is

illustrated in a representative histogram from a representative control puppy in *Figure 5b*. The ratio of mean channel fluorescence intensity between neutrophils and lymphocytes dropped in controls (0.62±0.2) while in vaccinates remained constant (1.10±0.11). This was not a global downmodulation of all cell surface proteins on neutrophils because expression of the protein bound by GM-1 antibody, present on 98% of neutrophils, remained constant throughout the study (*Figure 5c* and *d*).

To illustrate the change in expression of CD4 on neutrophils, the percentage of neutrophils whose CD4 levels were below that on lymphocytes was recorded at each timepoint for every animal (*Figure 6*). Significant differences in CD4 levels between neutrophils and lymphocytes was demonstrated for all control and one vaccinated animal beginning day 3. Neutrophil CD4 expression in nonvaccinated controls is statistically different from vaccinates ($P$=0.0001) on days 5 and 7 post-challenge. The reduced level of CD4 on neutrophils on day 7 negatively correlated and was statistically significant ($P$<0.05) with clinical scores ($r$=−0.79) and virus shed ($r$=−0.61) on day 8 post-challenge.

**Figure 6** Percent neutrophils having decreased CD4 expression on neutrophils when compared to lymphocytes is shown for vaccinated (●) and control puppies (○) after CPV-2b challenge. Group means are shown as a solid line. For individual animals, the lower limit cutoff for significant deviation from normal (group mean minus 2 S.D.) is indicated by a dotted line

## DISCUSSION

These studies clearly demonstrated that information obtained by measuring nonspecific products of inflammation or leukocyte trafficking by flow cytometry can effectively complement traditional methods of vaccine efficacy assessment. It was crucial to the success of these studies to determine whether these parameters correlated with traditional signs of the clinical disease. Thus, it was necessary for the CPV2b challenge virus used in this experiment to elicit severe clinical signs allowing clear association of each nontraditional parameter with clinical signs and virus shed. Elevated a-1AG and SAA levels occurred at the same time that puppies developed clinical signs and virus appeared in feces. Furthermore, the quantity of a-1AG or SAA measured in individual samples correlated with the severity of clinical signs and virus shed. Peak a-1AG levels coincided with the drop in total leukocytes on day 7. Only one puppy did not have elevated levels of either a-1AG or SAA post-challenge. This puppy died 6 days post-challenge and showed other signs of infection (i.e. lymphopenia, virus shed, and a drop in neutrophil-CD4 expression). The other two puppies had a delayed response to virus challenge. They did not show clinical signs until days 6 and 7 post-challenge, and virus shed did not begin until day 8, at which time SAA levels became elevated. Thus, in these instances, elevated production of acute phase proteins closely followed the appearance of virus shed in feces and development of clinical signs. When everything is considered, these data demonstrate that after CPV-2b challenge, elevated production of a-1AG or SAA, as well as changes in CD4-neutrophil expression, correlate with

and provide quantifiable indicators of virus infection and clinical disease.

Lymphopenia and neutropenia are important hematological changes described for natural infections of canine parvovirus, and a strong correlation between disease outcome and severity of neutropenia has been observed[12]. In experimental infections lymphopenia is most commonly documented while neutropenia is inconsistently demonstrated[28]. In both instances, laboratory assessment of these changes by manual differential count is inherently variable and subjective. This study demonstrated that by using antibodies and flow cytometric analysis to positively identify crucial cell populations, a more accurate, less variable depiction of leukocyte trafficking can be obtained. With flow cytometric analysis lymphopenia was documented for the majority of controls and none of the vaccinates. Furthermore, we were able to demonstrate that neutropenia followed neutrophilia in a significant number of controls and this pattern was not observed in the vaccinated animals. Thus, this methodology more accurately portrayed the clinical picture most commonly seen during natural infections.

In studies of natural infection, the direct or indirect effects of CPV on neutrophils were not examined because the neutropenia was attributed to exhaustion of neutrophils as a result of secondary bacterial infections[29]. The observations made in the present study are significant, therefore, because they demonstrate that CPV infection produces phenotypic and trafficking changes in neutrophils before neutropenia occurs. These data suggest that impaired neutrophil function occurs

as a direct or indirect effect of CPV infection. CPV may be the predisposing factor for secondary bacterial infections in puppies.

The changes in appearance of CD4 on canine neutrophils is puzzling. CD4 is most commonly found on a subset of T cells and, on those cells, participates in cell signalling during antigen recognition[30]. Canine and related carnivores are unique compared to other mammalian species because 95% of neutrophils as well as the T cell subset express high levels of the CD4 protein[31]. There is no known function for CD4 on neutrophils, and the significance of a change in level of neutrophil CD4 expression in this disease is unknown. However, the changes in CD4 expression and cell trafficking of neutrophils observed after CPV challenge implicate a role for these cells in pathogenesis of CPV disease. The significance of these changes should be emphasized considering that alterations in CD4 expression were observed only after viral infection, and that vaccination protected from these effects. It is also noteworthy that among the parameters evaluated in this study, the loss of neutrophil-CD4 expression was one of the most consistent findings and occurred in all the nonvaccinated controls, and only in one vaccinate. Several possibilities could account for the loss of CD4 and for the neutrophilia/neutropenia pattern observed in the controls. One possibility is a direct effect of CPV infection on bone marrow neutrophil precursor maturation. Changes in homeostasis may have resulted in export of immature neutrophils into circulation[32]. In support of this claim is the neutrophilia present at the time that CD4-neutrophil expression was low, however, the visual cell differential analysis did not reveal an increase in immature (band) neutrophils to account for the observed neutrophilia, and neutrophilia was observed in several vaccinates in the absence of any CD4-modulation. Thus, the changes in neutrophil-CD4 phenotype and trafficking may be an indirect effect of virus infection. Destruction of gut crypt cells and release of LPS and other bacterial products are known to induce IL-1, TNF-α (tumor necrosis factor-alpha), IL-8, and γ-interferon[33,34]. IL-1 is known to induce neutrophilia and in synergy with TNF will induce changes in vascular endothelium resulting in up or down regulation of cell receptors responsible for cell margination[35–37]. IL-8 is a neutrophil chemotactic factor which will activate and recruit neutrophils to sites of tissue damage[38], perhaps by drawing on bone marrow reserves of mature neutrophils as has been postulated for this disease[28,29]. CD4-levels on mature bone marrow derived neutrophils has not been examined, but may be present in lower quantities than on circulating neutrophils, accounting for the change in CD4 expression we observed. Regardless, the kinetics of the neutrophil-CD4 modulation and trafficking argue that it is the virus and not secondary infections that effect these changes. Furthermore, these changes may be a clue indicating pathogenic insult to neutrophil function.

Vaccination with the experimental CPV-2 vaccine effectively stimulated a SN response in all puppies. Since this response was measured against the CPV-2a strain, it supports previous data indicating that CPV-2 contains all the neutralizing epitopes of CPV-2a[2,3]. It is important to note that seroconversion occurred at different points in the three dose regimen and did not necessarily correlate with pre-existing titer. Three puppies did not sero-

convert until after the third vaccination and in one of these puppies, the SN titer did not increase after experimental infection with CPV-2b. Nonetheless, this animal remained normal, and did not exhibit any hematological changes, nor was virus shed in the feces. This observation is significant because this individual may represent a minor population of SN low responders, and suggests that local and cell mediated immunity may be sufficient to develop significant immune protection to CPV[39].

In summary, by studying the inflammatory events resulting from parvovirus infection we have identified quantitative nontraditional parameters that will complement traditional methods of evaluating vaccine efficacy. In so doing, we successfully demonstrated that puppies vaccinated when they were CPV seropositive were protected from the inflammatory and clinical consequences of parvovirus infection after a virulent CPV-2b challenge.

## ACKNOWLEDGEMENTS

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