Changes in Blastogenic Responses of Lymphocytes and Delayed Type Hypersensitivity Responses after Vaccination in Dogs

Tadashi MIYAMOTO, Yasuho TAURA, Satoshi UNE, Makoto YOSHITAKE, Sanenori NAKAMA, and Seiji WATANABE¹⁾

Department of Veterinary Surgery, Faculty of Agriculture, Yamaguchi 753 and ¹⁾Kyoritsu Shoji Co., Ltd., Chiyoda-ku, Tokyo 102, Japan

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ABSTRACT. To clarify the immunologic effects of vaccination in dogs, we monitored total leukocyte and lymphocyte counts, humoral antibody responses, blastogenic responses of lymphocyte, and delayed type hypersensitivity (DTH) responses after vaccination. Mixed vaccines were administered on day 0 except for canine parvovirus (CPV) vaccine which was readministered on day 21. The puppy and adult dogs had a significant decrease in leukocyte and lymphocyte counts on day 7. The puppies showed a significant increase in the blastogenesis of lymphocytes after each vaccination, whereas the adult dogs had no significant changes. However, the adult dogs were divided into two groups, high responders and low responders in blastogenesis of lymphocytes. The dogs with higher or lower response in SI values on day 0 tended to show decrease or increase after the first vaccination, respectively. Since almost all dogs developed high titers of humoral antibody, it is considered that vaccination acts in an immunomodulative fashion. DTH responses to phytohemagglutinin (PHA) and CPV vaccine monitored at 0, 3, and 8 weeks after the first vaccination produced strong reactions, in particular those to CPV vaccine rose significantly after vaccination and maintained the higher responses for at least 2 months. These results suggest that DTH responses to PHA and CPV vaccine are helpful to monitoring non-specific and specific immune functions *in vivo*, therefore, DTH could be used as simple and rapid immunologic tests in canine practice.—**KEY WORDS:** blastogenic responses of lymphocyte, delayed type hypersensitivity, dog, parvovirus, vaccination.

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Various immunologic assays have confirmed the induction of immunosuppression by general anesthetics and surgical procedures [4, 7, 14, 16]. With an increased susceptibility to infections by the stress of anesthesia and surgical procedures, nonvaccinated dogs may succumb to certain viral infectious diseases. To prevent such nosocomial infections, the vaccination close to the time of surgery in nonvaccinated dogs has been discussed.

There have been few studies on cell mediated immunity after canine parvovirus (CPV) vaccination [2, 13]. Several studies have emphasized the induction of immunosuppression by CPV [8, 10], however there is lack of definitive evidence to resolve the issue of immunosuppression by CPV vaccination. This evidence has not been obtained hitherto, probably because cell mediated immunologic assays and estimates have been difficult to carry out, in some respects, at surgery. By delayed type hypersensitivity (DTH) using the ear in our previously investigation, strong reactions were produced at the inoculation site with phytohemagglutinin and CPV vaccine [18]. Therefore, DTH in the ear seems to be useful in the determination of cell mediated immunity in dogs.

In this study, we attempted to clarify the immunologic effects of vaccination in healthy dogs by monitoring leukocyte and lymphocyte counts, humoral antibody responses, blastogenic responses of lymphocytes, and DTH responses.

MATERIALS AND METHODS

Animals: Nonvaccinated 6 beagle and 7 mongrel puppies with the age of 2–3 months old (puppy group) and 6 beagle and 6 mongrel adult dogs with the age from 1 to 5 years old (adult group) were used. All the dogs were clinically normal and were individually isolated for the duration of the experiment.

Vaccination: The vaccine used in this study was a mixture with inactivated CPV vaccine, modified live virus (MLV)-canine distemper virus (CDV) and MLV-canine adenovirus (CAV) 1 vaccine (Dohyvax DHP®, Duphar B. V., Weesp, Holland). All dogs were first administered subcutaneously with inactivated CPV vaccine in combination with MLV-CDV and MLV-CAV 1 vaccine, and readministered with CPV vaccine alone at 21 days after the first vaccination.

Blood sampling: Blood samples were collected at 0, 3, 7, 14, 21, 24, 28, 35, and 56 days after the first vaccination and the following analyses were performed.

Leukocyte and lymphocyte counts: Total circulating leukocyte counts were determined using a micro cell counter (CC-110, Toa Medical Electronics Co., Ltd., Kobe, Japan) and the absolute differential count for each sample calculated from a standard 200 cell count performed on a Giemsa-stained blood smear.

Serum antibody titers: CPV serum antibody titers were assayed by using the hemagglutination inhibition (HI) test [17]. The HI titer was recorded as the reciprocal of the highest dilution of serum showing complete inhibition of hemagglutination.

CDV serum antibody titers were assayed by micro-neutralization (SN) test [1]. SN tests were performed on Vero cells and the SN titer was calculated as the reciprocal of the highest serum dilution that neutralized viral infection and induced cytopathic effects in 2 of 3 replicate cultures.

Blastogenic responses of lymphocytes: The procedure for determining the blastogenic responses of lymphocytes was a modification of previously described ethidium bromide fluorescence assays [6, 18]. Peripheral blood lymphocytes were harvested by Ficoll separation (SG: 1.077, 17-0840-03 Pharmacia, New Jersey, U.S.A.) and washed twice in phosphate buffered saline (PBS, pH 7.2), after which the procedure was repeated with RPMI 1640 (05911 Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing fetal calf serum (FCS, Bioproducts, Maryland, U.S.A.). After the final washing, the suspensions were adjusted to a concentration of 5×10^5 cells/ml in the culture medium, which consisted of RPMI 1640 supplemented with 8% heat-inactivated FCS, pyruvic acid (1.2 mM), L-glutamine (3.4 mM), 2-mercaptoethanol (0.05 mM), penicillin (0.5 mg/ml), streptomycin (0.5 mg/ml), gentamicin (0.05 mg/ml), HEPES buffer (25 mM), and sodium bicarbonate (24 mM). The suspension was dispensed in 0.2 ml amounts into 96-well U-bottomed microtiter plates (3910, Falcon, Oxnard, U.S.A.). Then 0.01 ml of mitogen was added to each well, except for the control, to give a final concentration of 5 μ g/ml of mitogen. Phytohemagglutinin-P (PHA, Difco Laboratories, Michigan, U.S.A.) was used as a mitogen. Tests were done in quintuplicate. Cultures were maintained at 37°C under 5% CO₂ in air for 3 days. Wells were washed

3 times with PBS, and then 0.1 ml of PBS and 0.05 ml of sodium dodecyl sulfate (0.5 mg/ml, Wako Pure Chemical Industries Ltd., Osaka, Japan) were added to each well. Plates were incubated for 30 min at room temperature, and then 0.05 ml of ethidium bromide (0.02 mg/ml, Sigma Chemical Co., St. Louis, U.S.A.) was added to each well before the final incubation for 15 min at 24 °C. Fluorescence intensity was measured with a microplate reader (MTP-32, Corona Electric, Ibaraki, Japan). The blastogenic responses of lymphocytes were expressed as a stimulation index (SI), which was defined as the ratio of experimental to control intensity.

Delayed type hypersensitivity (DTH): Inoculations to determine DTH were performed at 0, 3, and 8 weeks after the first vaccination. All dogs were intradermally inoculated in the ear with 100 μ l CPV vaccine for specific immunity (DTH-CPV), with 100 μ l PHA for nonspecific immunity (DTH-PHA), and with 100 μ l physiological saline as a negative control [18]. At 24 and 48 hr after the inoculation, the DTH response of induration of the skin was measured at the inoculation site with a thickness gauge (Mitsutoyo, Tokyo, Japan) [18].

Statistical analysis: The results were expressed as mean values $\pm$ S.E. The statistical significance of the differences between the means of the sample groups was determined by Student's *t* test. P values of less than 0.05 were considered significant.

RESULTS

Changes in leukocyte and lymphocyte counts after vaccination: The changes in total leukocyte and lymphocyte counts for each group are shown in Fig. 1. Dogs of both groups showed a significant decrease in leukocytes counts on day 7. The puppy had a significant decrease in lymphocytes counts on day 7, and the adult dogs had a significant decrease in the number of lymphocytes on days 3 and 7.

Serum antibody titer test after vaccination: The changes in CPV and CDV serum antibody titers for each group are shown in Fig. 2. CPV serum antibody titers of the adult dogs were higher than those of the puppy on day 0. Both groups had a significant increase on day 7. Secondary immune responses were observed in both groups following the second CPV vaccination. CDV serum antibody titers rose more slower than CPV serum antibody titers, but both groups had enough production of both CPV and CDV antibodies, as determined by

Fig. 1. Changes in leukocyte (A) and lymphocyte counts (B) after vaccination. Each value represents the mean $\pm$ S.E. $\bullet$ — $\bullet$: puppy group, $\bullet$ --- $\bullet$: adult group. Significant differences. *: $P < 0.05$, **: $P < 0.01$.

Fig. 2. Changes in canine parvovirus (A) and canine distemper virus (B) serum antibody titers after vaccination. Each value represents the mean $\pm$ S.E. $\bullet$ — $\bullet$: puppy group, $\bullet$ --- $\bullet$: adult group. Significant difference. *: $P < 0.05$.

Fig. 3. Changes in blastogenic responses of lymphocytes to phytohemagglutinin after vaccination. Each value represents the mean $\pm$ S.E. $\bullet$ — $\bullet$: puppy group, $\bullet$ --- $\bullet$: adult group. Significant differences. *: $P < 0.05$ (compared with day 0), #: $P < 0.05$ (compared with the adult group).

Fig. 4. Changes in blastogenic responses of lymphocytes to phytohemagglutinin in the adult group after vaccination. Each value represents the mean $\pm$ S.E. $\circ$ — $\circ$: high responder group, $\circ$ --- $\circ$: low responder group. Significant differences. *: $P < 0.05$, **: $P < 0.01$ (compared with day 0), ##: $P < 0.01$ (compared with the high responder).

Fig. 5. Changes in delayed type hypersensitivity responses to phytohemagglutinin (A) and canine parvovirus (B) vaccine after vaccination. Each value represents the mean $\pm$ S.E. □: puppy group, ▨: adult group. Significant differences. *: $P<0.05$, **: $P<0.01$ (compared with the same hr at 0 week), #: $P<0.05$, ##: $P<0.01$ (compared with the adult group).

the HI and SN tests, respectively.

Blastogenic responses of lymphocytes after vaccination: Changes in the blastogenic responses of lymphocytes to PHA for each group are shown in Fig. 3. After each vaccination, the puppy showed a rapid increase in SI values. In particular, these values were significantly higher on days 14, 21, 24, and 35. On the contrary, there was no significant change in SI values after any vaccination in the adult group, however, the adult group could be divided into two further groups according to reaction after the first vaccination (Fig. 4). Half of the dogs with lower SI values on day 0 showed an increase in SI values after the first vaccination (high responder group), whereas the other half of the dogs with higher SI values on day 0 showed a decrease in SI values after the first vaccination (low responder group). The difference in SI values on day 0 between both groups (high responder and low responder) was significant. The low responder group had significantly higher SI values than the high responder group on day 0. The dogs that had low SI values on day 0 tended to exhibit increased SI values following the first vaccination, whereas the dogs that had high SI values on day 0 tended to exhibit decreased SI values following the first vaccination. Regarding the blastogenic responses of lymphocytes to PHA, the puppy group had significantly lower SI values than the adult group on day 0.

DTH responses after vaccination: No responses were initiated by challenge with physiological saline, whereas CPV vaccine and PHA produced strong reactions (Fig. 5). Regarding DTH-PHA responses, the puppy group showed weaker reactions than the adult group. DTH responses at 0 week and 48-hr responses at 8 weeks in the puppy group were

significantly weaker than those of the adult group. In the puppy group, DTH-PHA 24-hr responses at 3 weeks were significantly stronger than 24-hr responses at 0 week. DTH-CPV responses at 3 and 8 weeks in both groups were significantly stronger responses at 0 week. DTH-CPV responses in both groups were similar.

DISCUSSION

Several studies have emphasized the induction of immunosuppression by CPV vaccination basing their conclusions on the following assumptions: (1) CPV was the common factor in the occurrence of lymphopenia [3, 12, 15] and (2) there was a depletion of lymphocytes from the lymph nodes and thymus of dogs infected with virulent CPV [2, 15]. But little has been studied cell mediated immunity after CPV vaccination [2, 13].

DTH is a T cell-dependent immune phenomenon manifested by an inflammatory reaction at the site of antigen deposition, usually at the skin. DTH reaches its peak intensity 24- to 48-hr after initiation. In this study, DTH-CPV responses increased after vaccination and maintained the higher levels for the 2 months' duration of the experiment. Increases in DTH-CPV responses tended to occur according to increases in CPV antibody titers, although there were slight differences between the two responses. Since DTH-CPV responses can be induced easily and rapidly, it could be used for testing the effect of CPV vaccine in the field, similar to the DTH-tuberculin test.

The changes in DTH-PHA responses resembled those in the blastogenic responses of lymphocytes to PHA. Since the procedures for DTH-PHA re-

sponses were also quite easy, thus these responses could be used as a simple nonspecific cell mediated immunologic test *in vivo*.

Transient lymphopenia may occur after vaccination with some MLV-CPV vaccines [2, 8] and this observation has resulted in the ideas that MLV-CPV vaccines are immunosuppressive and that vaccines containing MLV-CPV in mixture with MLV-CDV, CAV, and canine parainfluenzavirus are not necessarily safe [10]. However, another study has shown that this transient lymphopenia may be indicative of an active immune response to the vaccine and that it may represent changes in the dynamics of the circulation and migration of sensitized lymphocytes in response to the replication of vaccine virus in the dog [2]. In our investigation, both puppies and adult dogs showed significant decreases in the number of leukocytes and lymphocytes on day 7 following vaccination and remarkable fluctuations in the blastogenic responses of lymphocytes on days 3 and 7. Thus, the vaccinated dogs might have experienced a relative hematologic and immunologic imbalance for 3 to 7 days after the first vaccination, prior to the production of antibodies. However, the findings that almost all dogs developed high titers of humoral antibody and that almost all dogs in the puppies and half of the adult dogs had increased SI values with regard to blastogenic responses of lymphocytes after the first vaccination suggest that this leukopenia and lymphopenia may not be as immunosuppressive as has been assumed by Kesel and Neil [8]. Furthermore, the dogs with higher or lower response in SI values on day 0 tended to show decrease or increase, respectively. It is considered that vaccination may act in an immunomodulative rather than an immunosuppressive fashion, depending on the immunity of responder. However, further studies are needed to clarify this situation.

Antibody titers rose more slowly in the puppy group than in the adult group. In addition, the blastogenic responses of lymphocytes on day 0 in the puppy group were lower than those in the adult group, and DTH-PHA responses in the puppy group were lower than those in the adult group. It has been reported that the immune response of puppies does not approximate that of adult dogs until puppies are about three months of age [5, 9, 11]. Similarly to these reports, our present study suggests that the immune system and other host defenses may not be fully developed in puppies that are 2 to 3 months old.

As conclusion, these findings suggest that DTH may be used as simple immunologic tests and vaccination may not act in immunosuppressive fashion. To prevent nosocomial infections, vaccination a few weeks before surgery is necessary. However, in nonvaccinated patients, investigation for the effects of vaccination on immune responses close to the time of surgery in various situations will be needed by various immunologic tests including DTH.

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