VACCINE-INDUCED AUTOIMMUNITY IN THE DOG

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I. Introduction

Vaccines are widely used in human and veterinary medicine as an effective and economic method to control viral and bacterial diseases. Although generally considered safe, vaccination is occasionally accompanied by adverse affects. Many adverse affects related to vaccination are acute and transient, for example, fever, swelling at the site of the inoculation, and allergic reactions. In contrast, reports of autoimmune disease following vaccination are relatively rare. In most instances, it is difficult, if not impossible, to ascertain that vaccination caused or precipitated the autoimmune disease. In a recent report, the Advisory Committee on Immunization Practices in people concluded that there is a causal relation between diptheria-tetanus-pertussis (DTP) and measles-mumps-rubella (MMR) vaccination and arthritis, but no evidence of a causal relationship between these vaccinations and other autoimmune diseases such as autoimmune hemolytic anemia and Guillain-Barre syndrome (Centers for Disease Control and Prevention, 1996). Cohen and Shoenfeld (1996) also stated that the relation between vaccination and autoimmunity is obscure. They added that there is a need for experimental studies to address this subject (Cohen and Shoenfeld, 1996).

There has been a growing concern among dog owners and veterinarians that the high frequency with which dogs are being vaccinated may lead to autoimmune and other immune-mediated disorders (Dodds, 1988; Smith, 1995). The evidence for this is largely anecdotal and based on case reports. A recent study observed a statistically significant temporal relationship

between vaccination and subsequent development of immuno-mediated hemolytic anemia (IMHA) in dogs (Doval and Ciger, 1996). Although this does not necessarily indicate a causal relationship, it is the strongest evidence to date for vaccine-induced autoimmune disease in the dog.

We are investigating the effect of vaccination on dogs in a series of experimental studies. The goals of these experiments are (1) to determine if vaccination of dogs affects the function of the immune system and, in particular, if vaccination results in autoimmunity; (2) to delineate the mechanisms by which vaccination results in autoimmunity if this occurs; and (3) to develop alternative vaccination strategies that will not be accompanied by adverse effects. The issue that is the focus of this and ongoing studies in our laboratory is somewhat different from that examined by Duval and Ciger (1996). In their study, a statistically significant temporal relationship between the onset of IMHA and prior vaccination suggested that vaccination caused IMHA or accelerated preexisting IMHA in adult dogs. Although not documented, it is likely that these middle-aged dogs had received multiple vaccines prior to the last vaccination. Why this last vaccination suddenly triggered the onset of IMHA is unknown. In contrast, our studies examine if vaccination of dogs at a young age causes alterations in the immune system, including the production of autoantibodies, that could eventually lead to autoimmune disease in susceptible individuals. In this paper, we report on the findings of the first study in which a group of vaccinated dogs and a group of unvaccinated dogs were followed for 14 weeks after the first vaccination.

II. Materials and Methods

A. Animals

Two pregnant Beagle dogs were purchased from a commercial breeder. The animals whelped in the Animal Facility of the Purdue University School of Veterinary Medicine and the pups were weaned at 6 weeks of age. Five pups were assigned to one of two groups, a vaccinated and an unvaccinated group, based on body weight, gender, and litter of origin. The vaccinated and unvaccinated group of dogs were housed in separate rooms.

The dogs were examined daily. Rectal temperature and body weight were recorded twice a week. Blood samples were collected from the jugular vein prior to each vaccination and 2, 5, 7, and 14 days following vaccination for hematology, endocrinology, and viral serology. Blood samples collected on days 5 and 14 following vaccination were also used for lymphocyte phenotyping and lymphocyte proliferation assays, and blood samples collect at 7 days following vaccination were used for the detection of autoantibodies.

B. Vaccination Schedule

The dogs in the vaccinated group were injected subcutaneously with a commercially available multivalent vaccine, Vanguard-5 CV/L (Pfizer, Croton, CT) at 8, 10, 12, 16, and 20 weeks of age according to the instructions of the manufacturer. They were injected subcutaneously with an inactivated rabies vaccine, Imrab-2 (Rhone-Mericux, GA) at 16 weeks of age. The unvaccinated group of dogs received subcutaneous injections of sterile saline at the same time points.

Both groups of dogs were injected subcutaneously with 1 mg of keyhole limpet hemocyanin (KLH, Calbiochem) in RIBI-adjuvant at week 20.

C. Viral Serology

Serum samples collected at 6 weeks of age and 0, 2, 5, 7, and 14 days after each vaccination were assayed for the presence of antibodies to canine distemper virus by serum neutralization test, and for antibodies against canine parvovirus by hemagglutination inhibition test. Serum samples were analyzed for antibodies against rabies virus at 16 and 20 weeks of age by a rapid fluorescent focus inhibition test.

D. Hematology

Blood samples were collected at 0, 2, 5, 7, and 14 days after each vaccination for hematocrit, corrected white blood cell count and differential, and platelet counts.
E. Endocrinology
Plasma and serum samples collected at 0, 2, 5, 7, and 14 days after each vaccination were assayed for curtisol, triiodothymonine (T3), and thyroxine (T4) by radioimmunoassay.

F. Immunology
Lymphocyte phenotyping was used. Whole blood was stained with a panel of mouse monoclonal antibodies, followed by F(ab')2 goat anti-mouse IgG (Jackson Research Laboratories). The monoclonal antibodies used were CA2.1D6 (anti-CD21), CA15.8G7 (anti-TCRoB), CA20.8H1 (anti-TCRv81, 12.125 (anti-CD4), and 1.140 (anti-CD8). The characteristics of these monoclonal antibodies have been described (Gebhard and Carter, 1992; Moore et al., 1995). Following red blood cell lysis and fixation in 2% paraformaldehyde, the cells were analyzed by flow cytometry.

G. Lymphocyte Blastogenosis Assay
Heparinized blood samples were diluted 1:10 in RPM1-1640 and distributed in the wells of a 96-well plate. Triplicate samples were incubated for 96 hours in the presence of medium only, 2.5 and 5 pg/ml PHA, 5 and 10 pg/ml Concenavalin A (Con A) and 1 and 10 pg/ml PWM. During the last 24 hours of incubation the wells were pubed with 0.5 uCi of H-thymidine. The cells were harvested with a 96-well cell harvester, and the incorporation of radioactivity was measured in a TopCount scintillation counter (Packard Instrument Co., Meriden, CT).

H. Enzyme-Linked Immunosorbent Assay (ELISA)
The presence of antibodies reactive with homologous and heterologous antigens in serum samples collected at 22 weeks of age was analyzed by an indirect ELISA. High-binding ELISA plates (Costar, Cambridge, MA) were coated with 10 pg/ml of antigen in 0.1 M bicarbonate buffer. The wells were rinsed and incubated for 1 hour with phosphate-buffered saline (PBS)/0.1% Tween. Serum samples were diluted 1:10 in PBS and added to the wells in triplicate. Following incubation, the wells were rinsed and incubated with alkaline phosphatase labeled goat anti-dog IgG (Kirkegnard and Perry, Gaithersburg, MD). Alkaline phosphatase activity was measured after addition of p-NPP substrate at 405 nm in a microplate reader (Molecular Devices, Menlo Park, CA).
Essentially the same procedure was used to measure the presence of antibodies against KLH. Alkaline phosphatase labeled anti-dog IgM and IgG were used as secondary reagents.

I. Necropsy
At 22 weeks of age, the dogs were killed by intravenous injection of barbiturates, and a complete necropsy performed. Tissue samples were collected in 10% buffered formalin and processed for light microscopic examination. The tissues that were examined included the spleen, lymph nodes, tonsils, thymus, Psyer's patches, adrenal glands, thyroid glands, pituitary gland, pancreas, heart, lung, kidney, liver, and brain.

J. Statistical Analysis
Data were analyzed for significant differences between groups by Student's t test or repeated measures ANOVA and a significant change over time using a repeated measures ANOVA.

III. Results

A. Viral Serology
None of the pups had detectable antibodies against canine distemper virus and canine parvovirus at 6 weeks of age and against rabies virus at 16 weeks of age. The unvaccinated

dogs remained seronegative for these three viruses during the course of the study. The dogs that were immunized developed titers against CDV (maximum titers ranged from 1:48 to 1:1024), CPV-2 (1:320 to 1:1280), and rabies (1:25 to 1:1000).

B. Clinical Observations, Hematology, and Endocrinology

No differences between the unvaccinated and vaccinated groups were found for rectal temperature, body weight, and hematologic values.

There were no significant differences between unvaccinated and vaccinated dogs for concentrations of cortisol, T3, and T4. However, a significant ($p<0.02$) change was observed over time for each of these three hormones. The plasma concentration of cortisol decreased from a mean of 41.1 ng/ml at 8 weeks of age to 17.6 ng/ml at 22 weeks of age. The concentration of T4 also decreased, from 31.1 ng/ml at 8 weeks of age to 22.8 ng/ml at 22 weeks of age. The concentration of T3 increased from 0.63 ng/ml at 8 weeks of age to 1.1 ng/ml at 22 weeks of age.

C. Immunology

No differences were observed unvaccinated and vaccinated dogs for lymphocyte subpopulations or for the proliferative response to any of the mitogens tested.

The response of both groups of dogs to KLH was similar. There was no statistically significant difference in the KLH-specific IgM and IgG concentrations in the serum (not shown).

At 8 weeks of age, antibodies against homologous and conserved heterologous antigens were negligible in the serum of the dogs. At 22 weeks of age there was a significant increase of IgG antibodies reactive with 10 of 17 antigens in the vaccinated dogs versus no increase in the unvaccinated dogs (Table I). The increase of optical density was modest for 8 of these 10 antigens, but a large increase was observed for fibronectin and laminin. All vaccinated dogs developed high levels of fibronectin-specific IgG antibodies. Similar levels of IgG anti-fibronectin antibodies were observed when bovine fibronectin was substituted by human or mouse fibronectin (not shown). The concentration of anti-fibronectin antibodies began to increase after the second vaccination in three dogs and after the third vaccination in the other two vaccinated dogs, and reached a maximum level after the fourth vaccination (Fig. 1). To determine if the antibodies had a preferential reactivity with a particular part of the bironectin molecule, we tested the reactivity of serum samples with two fragments of the fibronectin. The 30-kDa fragments contains the heparin-binding domain of fibronectin, whereas the 45-kDa fragment contains the collagen-binding domain. As shown in Fig. 2, little reactivity was observed with the 45-kDa fragment, but significant reactivity was observed with the 30-kDa fragment.

High levels of anti-laminin antibodies were observed in the serum of three of the five vaccinated dogs at 22 weeks of age. One dog had high levels at 17 weeks of age, whereas the other two dogs did not devlop high levels until the end of the study.

High levels of antibodies reactive with skeletal muscle myosin and myoglobin were observed in both groups of dogs at 22 weeks of age. The antibody levels increased at 11 weeks of age in three dogs, at 13 weeks of age in another three dogs, and at 17 weeks of age in the remaining four dogs.

D. Necropsy

Gross and light microscopic examination of the tissues of the dogs revealed no significant lesions. The thyroid gland of one of the vaccinated dogs had a small lymphoid nodule with obliteration of adjacent thyroid follicles.

IV. Discussion

In this study, we exhaustively evaluated the effects of vaccination with a multivalent vaccine

and a rabies vaccine on the immune system of young dogs. Vaccination did not cause immunosuppression or alter the response to an unrelated antigen (KLH). In contrast to an earlier study (Mastro et al., 1986), but in agreement with other work (Phillips and Schultz, 1987), we did not observe a transient lymphopenia in the dogs at any time. However, vaccination did induce autoantibodies and antibodies to conserved heterologous antigens. The pathogenic significance of these autoantibodies is presently uncertain. We did not find any evidence of autoimmune disease in the vaccinated dogs, but the study was terminated when the dogs were 22 weeks of age, well before autoimmune diseases usually become clinically apparent. It is likely that genetic and environmental factors will trigger the onset of clinical autoimmune disease in a small percentage of the animals that develop autoantibodies. For practical and economic reasons, only a small number of dogs can be followed in an experimental study, and clinical autoimmune disease may, therefore, never be observed. The principal value of an experimental study is that it enables us to determine the frequency of autoantibody responses and the mechanism(s) that cause vaccines to induce autoantibodies. We used two vaccines, a multivalent vaccine and an inactivated rabies vaccine of a particular commonly used brand. We consider it unlikely that the observed autoantibodies were specifically induced in response to those brands of vaccine and this phenomenon will likely occur with other commercial vaccines. In a follow-up study, we have observed similar autoimmune phenomena in dogs immunized with the multivalent vaccine only and in dogs immunized with the rabies vaccine only (unpublished observations).

There was a marked increase of autoantibodies to the skeletal muscle proteins, myoglobin and myosin, in both groups of dogs. The reason for the appearance of these antibodies is uncertain, but it may be the result of the frequent blood sampling of the dogs. The dogs were bled five times following each vaccination, and some tissue trauma was unavoidable.
We examined the thyroid and adrenal cortical function in the dogs, and did not find evidence of any abnormality. Autoimmune thyroiditis is one of the most common autoimmune diseases of dogs, and it has been suggested that the apparent increase of this condition in dogs is related to the increased frequency of vaccination with modified live vaccines. There was no increase of anti-thyroglobulin antibodies in the vaccinated animals, or other evidence of thyroid dysfunction. However, the lymphoid nodule found in the thyroid gland of one of the vaccinated dogs may be an early manifestation of thyroiditis, a common lesion in purpose bred Beagles (Fritz et al., 1970).

The most strikingly increased concentrations of autoantibodies were directed against fibronectin and laminin. Fibronectin is widely distributed in the body as a component of the extracellular matrix and plasma. The anti-fibronectin antibodies were reactive with fibronectin of bovine, murine, and human origin. Although we have not yet demonstrated that they also react with canine fibronectin, this is very likely, since fibronectin is highly conserved between species. Anti-fibronectin antibodies have been found in human patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis, and a patient with a poorly defined connective tissue disease (Henane et al., 1986; Atta et al., 1994, 1995; Girard et al., 1995). The anti-fibronectin antibodies in four human SLE patients were directed against the collagen-binding domain (Atta et al., 1994), in contrast to the anti-fibronectin antibodies in the vaccinated dogs, which showed no affinity for this domain. The anti-fibronectin antibodies in the human patient with connective tissue disease showed reactivity with the cell-binding domain of fibronectin (Girard et al., 1995).

Anti-fibronectin antibodies have been experimentally induced in rabbits by immunization with human fibronectin in complete Freund's adjuvant (Murphy-Ullrich et al., 1984). The antibodies were reactive with both human and rabbit fibronectin. The rabbits subsequently developed a glomernlopathy with granular deposits suggestive of immune complexes in the glomcrular basement membrane. Anti-fibronectin antibodies have been induced in mice by multiple injections of homologous fibronectin without adjuvant (Murphy-Ulrich et al., 1986). The titer of

anti-fibronectin antibodies was much lower in mice immunized with native fibronectin than in mice immunized with de-natured fibronectin. However, in both groups, immune complexes were present in the serum and in the glomerali (Murphy-Ullrich et al., 1986). Light microscopic examination of the glomerali of the kidneys of vaccinated dogs did not reveal evidence of glomerunfpathy, but we cannot exclude the possibility of sub-light microscopic lesions. Anti-laminin antibodies were prevalent in the serum of three of the five vaccinated dogs. Anti-laminin antibodies are increased in human patients with SLE, rheumatoid arthritis, and vasculitis. Injection of polyclonal anti-laminin antibodies into rats resulted in glocnerulopathay and proteinuria (Abrahamson and Caulfield, 1982)> Anti-laminin antibodies have also been implicated in glomaerular disease in rats induced by mercuric chloride (Aten et al., 1995).

The mechanisms that may underlie the production of autoantibodies following vaccination are unknown, but at least four mechanisms can be proposed: cross-reactivity with vaccine-components, somatic mutation of immunoglobulin variable genes, "bystander activation" of self-reactive lymphocytes, and polyclonal activation of lymphocytes. Perhaps the simplest and most likely mechanism is that of cross-reactivity of vaccine and self-antigens. (Schattner and Rager-Ziaman, 1990), the most likely sources of cross-reactive epitopes are bovine serum and cell culture components. These are present in almost all vaccines as residual components of the cell culture necessary to generate vaccine viruses and may purposely be added to the vaccine as a stabilizer. In the presence of an adjuvant, these bovine products stimulate a strong immune response and induce antibodies that cross-react with conserved canine antigens. Thus, the strong response to fibronectin in the vaccinated dogs is most likely the result of the injection of bovine fibronectin contaminants in the vaccine. Indeed, this is essentially identical to the protocol used to produce anti-fibronectin antibodies in rabbits with human fibronectin in complete Freund's adjuvant (Murphy-Ullrich et al., 1984), as mentioned above. The lower response to other antigens (e.g., cardiolipin and laminin) may be due to a lower concentration of these antigens in the vaccine or lower immunogenicity.

During every immune response, self-reactive B and T lymphocytes are generated and activated. This is the result of somatic mutation and bystander activation. Under normal conditions, this will not lead to significant production of autoantibodies, because of the selection process in the germinal centers of lymph nodes. In the germinal centers only B cells that successfully compete for interaction with antigen presented on the surface of follicular dendritic celia will be allowed to survive (MacLennan, 1994). These B cells generally have high-affinity receptors for the antigen to which the immune response was induced. B cells with low affinity for the antigen or affinity for other antigens, including self-antigens, will undergo programmed cell death. The B cells with high-affinity receptors express bc1-2, which may rescue them from programmed cell death (MacLennan, 1994). This mechanism was elegantly demonstrated in mice immunized with a nominal antigen phosphorylcholine (Ray et al., 1996). A single point mutation in the hypervariable region of the expressed immunoglobulin genes was sufficient for the phosphorylcholine-specific B cells to acquire specificity for DNA. However, it was only possible to demonstrate DNA-specific B cells by fusing germinal center B cells with celia that expressed high levels of bc1-2, thereby rescuing them from programmed cell death (Ray et al., 1996). An increased expression of bc1-2 was observed in thymic lymphoid follicles of patients with myasthenia gravis, suggesting that failure to delete self-reactive B cells in these patients may lead to autoimmune disease (Shiono et al., 1997). While this may seem an attractive hypothesis to explain autoimmune phenomena in human beings and dogs, there is currently no evidence that this is a common mechanism.

Finally, polyclonal activation of lymphocytes, including activation of self-reactive lymphocytes, is a possible mechanism of vaccine-induced autoimmunity. Certain viruses and bacteria have superantigen or mitegen activity (Schwarts, 1993). This could also be the case for the microbial products included in the vaccines. The present study does not support this mechanism. Firstly, antibodies were observed against 10 of 17 antigens tested. Secondly, the

anti-fibronectin antibodies did not react with any portion of the fibronectin molecule, but instead, reacted most strongly with the heparin binding domain. These observations indicate that the appearance of autoantibodies in the serum of vaccinated dogs is an antigen-driven process and not caused by polyclonal activation. As argued earlier, the main antigens implicated are cell culture contaminants and bovine serum components.

In the dog, certain autoimmune diseases occur more frequently in particular breeds of dogs, indicating genetically determined susceptibility (Dodds, 1983; Happ, 1995). There is abundant evidence from studies in rodents and human beings that the magnitude of the antibody response and the susceptibility to autoimmune disease are in part genetically determined (Schwartz, 1993). It is likely that genetic factors also determine the susceptibility to vaccine-induced autoimmunity. That this is indeed the case is suggested by the finding that only three of the five vaccinated dogs developed a strong anti-laminin antibody response and that the kinetics of the anti-fibronectin response differed between individual animals. Identification of susceptibility genes will be important, because it may shed light on the pathogenesis of the autoimmunity. In addition, it will provide genetic tests that will enable dog breeders to monitor the susceptibility of their breeding stock to vaccine-induced autoimmunity.

Although the pathogenic significance of the vaccine-induced autoantibodies is still unclear, there are a number of ways to prevent their induction. Not vaccinating dogs is not a viable option, because the benefits of vaccination clearly outweigh the still uncertain risks of immune-mediated disease. However, since bovine serum components in the vaccine may be responsible for the majority of autoantibodies, elimination of these bovine components may avoid this problem. This could be accomplished by substituting homologous serum for bovine serum. However, as mentioned earlier, anti-fibronectin antibodies may still be induced by immunization with homologous fibronectin. New generations of vaccines, especially naked DNA vaccines, are free of serum components, and these should not induce autoantibodies. A recent study in mice indicates that DNA vaccination does not induce or accelerate autoimmune disease (Mer et al., 1997). Finally, mucosal vaccines are less likely to induce autoantibodies than parenterally administered vaccines. Depending on the formulation of the vaccine, soluble serum components are less likely to be absorbed via the mocosal surface, and, in fact, may induce tolerance instead of autoantibodies (Weiner et al., 1994).

In conclusion, we have demonstrated that vaccination of dogs using a routine protocol and commonly used vaccines, induces autoantibodies. The autoantibody response appears to be antigen driven, probably directed against bovine antigens that contaminate vaccines as a result of the cell culture process and/or as stabilizers. The pathogenic significance of these autoantibodies has not yet been determined.

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