Animal Protocol Form -- Aves Labs, Inc.

Protocol #: 04

Protocol Title: Production of egg yolk antibodies in chickens

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Signature of P.I.: _____

Date: _____

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Signature of Veterinarian: _____

Date: _____

Date of IACUC submission: 5 July 2008

Date of IACUC approval: _____

Name of IACUC Chair: William R. Woodward, Ph.D.

Signature of IACUC Chair: _____

Date: _____

1. **Background:** This protocol is meant to replace protocols #01, #02, and #03 which were approved by the IACUC at Aves Labs, Inc. in July 1999, June 2004, and June 2007, respectively. Although the basic features of these three protocols have not changed, these revisions serve to further explain and refine the underlying rationale. Specifically, the purpose of the first revision (i.e., June 2004) was (i) to update some of the details of the protocol, (ii) to incorporate modifications and addendums that have been approved by the IACUC over the years, and (iii) to up-date the format to make it compatible with that described on pages 9-15 of the pamphlet "Working with the IACUC: Writing an Animal protocol" published by the American Association for Laboratory Animal Science (ALAS) (2002). The purpose of the second (i.e., July 2007) revision is to further define the criteria used to assess pain and suffering in the animals, so that it is clear when an animal must be treated for pain, or euthanized. The purpose of the third (i.e., July 2008) revision was to limit the number of injections to which a hen would be subjected. Briefly, this upper limit is now defined at 10, with the understanding that additional injections could be made on an ad hoc case with the permission of a majority of IACUC members.

2. Description of the Project: This protocol describes the process by which egg yolk antibodies are produced in chickens for paying clients. It describes the collection of "preimmune eggs," the injection protocol, and the collection of "immune eggs" after the customary 4 injection series is completed. This protocol also includes some additional procedures that might be performed with some hens, as requested by clients. Specifically, these additional procedures might include additional injections beyond the customary four, and the collection of various tissues following euthanization of the hens.

2A. Collection of "Preimmune Eggs." Preimmune eggs are those collected from the hens prior to initiation of the injection protocol. In most circumstances, the hens are not handled during this period, and their eggs are simply collected. Since the cage floors are slightly slanted towards the front, the eggs roll down the floor to a shelf at the front of the cage under the food trough. Animal care technicians walk down the rows of cages, collecting the eggs and recording the cage numbers and dates on the shell of each egg using a #2 pencil. Once 3-4 pre-immune eggs are collected from a hen (usually over a 3-4 day period), the hen is then ready to undergo the injection protocol.

2B. Injection Protocol. Hens are injected a total of four times over a 7-8 week period, with injections typically performed every 2-3 weeks. These injections involve two animal care personnel -- one to hold the hen, the other to perform the injection itself. Additional injections may take place (i.e., beyond the standard set of 4 injections) when a particular project requires additional antibodies, but in the unlikely case that a given hen requires more than 10 injections, permission from a majority of IACUC members would be required prior to these injections. In expedited cases, the prior permission from Drs. Woodward and Lintner would both be required, but that full discussion of the circumstances of the project would be discussed at the next IACUC meeting.

Antigens. Antigens may consist of either (i) protein (recombinant or purified from a natural source), (ii) synthetic peptide covalently conjugated to carrier protein, such as keyhole limpet hemocyanin (KLH), (iii) small hapten molecule covalently conjugated to carrier protein, or (iv) mixtures of proteins. The preferred vehicle solution for these preparations is phosphate buffered (pH 7.2, 10 mM) isotonic saline solution (PBS). In some cases, other vehicle solutions might be substituted for PBS, such as Tris-buffered (pH 7.6, 10 mM) isotonic saline solution (TBS), or similar non-toxic buffers. Care is taken to avoid any buffer systems that might influence the behavior or health of the hens, such as cacodylate buffer, or any preparation that would contain toxic preservatives, such as thimerosal or azide. Similarly, care will be take to avoid any antigen that might influence the behavior or health of the hens. If there is any question about whether a particular antigen or vehicle might be a potential health hazard to the hens, the client is contacted by Dr. Ciment or one of the animal care technicians for more clarification.

Immunogens. Immunogens are prepared the day before injection and then stored at 4˚C. Immunogens are prepared by mixing 0.5 mls of antigen solution with 0.5 mls of adjuvant. The first injection utilizes Complete Freund's Adjuvant (CFA), while the second and third injections utilize a 1:1 mixture (v/v) of CFA and Incomplete Freund's Adjuvant (IFA), and the fourth injection utilizes IFA only. These two volumes are mixed by passage through a mixing chamber between two 3.0 ml sterile glass syringes. The syringes and mixing

chamber are connected by Luer locks. Once the immunogen has been transformed into a stable emulsion, the full volume of the immunogen (approximately 1.0 mls) is placed in one of the two syringes, and a 21 gauge needle is added to the end of the syringe, via its Luer-lock fitting. (NOTE: See Section 14 below for a justification for the use of Freund's adjuvants over other adjuvants)

Preparation for Injection. During the injection itself, the hen is removed from her cage by one of the animal handlers (the "holder"), and held with her back against that handler's chest with the hen's wings gently held back at the forelimb. In this way, the hen's pectoral area faces the second animal technician who will be performing the injections (the "injector"). When held firmly but gently in this position, the hen typically remains quiet, tending to avoid situations with sudden movements during the injection. A spray of 70% isopropanol is then applied to the pectoral region by the "injector," with care taken to avoid accidentally spraying near the hen's eyes. Then, using a sterile cotton pad soaked in 70% isopropanol, the feathers are moved out of the way exposing bare skin over the pectoral region. The hen is now ready for injection.

Injection. The "injector" performs a total of four injections into the pectoral muscles of the hen. Each injection is approximately one quarter of the total volume of 1.0 mls, or about 250 µl each. Two injections are placed into the right pectoral muscle and another two injections are placed into the left pectoral muscle. Care is taken to avoid accidentally nicking superficial veins, which can easily be seen below the surface of the skin. Once it is clear that the hen is not bleeding from one or more of the injection sites, the hen is replaced in her cage. If the hen <u>is</u> bleeding from one or more injection site, the "holder" continues to hold the hen, while the "injector" applies pressure to the site with a sterile cotton paid for a period of 10 seconds, or as long as it takes for the bleeding to subside. At this point, the hen is then replaced in her cage.

Intervals between injections. The schedule of injections is once every 2-3 weeks. Typically, the interval between the first and second injections is 3 weeks, while the interval between the second and third, and between the third and fourth, is 2 weeks. Depending on the time constraints, a client may require that their hens should be injected every 3 weeks. Although this adds an additional two weeks to the injection protocol, it is generally believed that the antibody titers are somewhat better, presumably because the stimulated B-cells have gone through their entire antigen-stimulation cellular program.

2C. Collection of "Immune eggs." The procedure for collecting "immune eggs" is very similar to that for "preimmune eggs" described above, except that this collection occurs <u>after</u> the injection series has been started. Typically, collection of "immune eggs" starts one week after the third injection and continues for four weeks after the fourth injection (or longer, if additional injections are performed). It is our experience that antibody titers in the egg yolks begins to increase about 5 days after injection and continues to increase for another 3-4 days, before starting to decrease. For this reason, collection of optimal "immune eggs" starts 7 days after the fourth injection and continues until a total of 18 eggs are collected (typically over a 3-week period). As more "optimal eggs" are collected, sub-optimal eggs are discarded.

2D. Additional Injections and Additional Egg Collection. One of the advantages for using chickens over mammal hosts for polyclonal antibody production is the option of being able to obtain large quantities of antibodies at a fraction of the cost. Consequently, some clients request that we perform additional injections of antigen beyond the typical four injection series, and that we collect additional batches of immune eggs. Typically, this involves two additional injections over a 3-week interval, followed by additional collections of eggs. In rare circum-stances, clients have us perform such additional injections over longer periods of time. In all cases, we work with the client to calculate the number of additional injections required and the number of additional batches of eggs to be collected, based on their results from the initial batches. If the total number of injections will exceed 10 into any given hen, prior permission from the IACUC will be required (see section 2B, above).

2E. Syringe Sterilization Protocol. Syringes are sterilized prior to injection by immersion in boiling water for a period of at least 10 minutes, followed by washing with non-ionic detergent, and exhaustive washing with deionized, sterile water to remove residual detergent. The syringes are then immersed in a bath of 70% isopropanol for at least 24 hours prior to drying and storage.

2F. Surgeries / Anesthesia / Physical Restraint. It should be noted this protocol does NOT permit surgeries on any animals at any time, it does NOT permit the use of anesthetic agents, and the only times the animals are physically restrained are momentary ones, while the animals are being held for injection or at the end of the project during euthanization. Moreover, this protocol NEVER permits the hens to be deprived of food or water to to ever be exposed to toxic materials.

3. **Justification for the Use of Animals**: Antibodies are critical reagents in a number of biochemical and biological applications. Their ability to selectively recognize specific gene products, even in the presence of closely related gene family members, offers scientists and clinicians critical tools for biomedical research and clinical diagnostics. The assays in which antibodies are used include western blots, ELISAs, immunostaining, immunoprecipitation, and various clinical tests for gene products associated with diseases in humans and animals.

   At the present time, there are no alternatives to the injection of animals for production of polyclonal antibodies. Although monoclonal antibodies offer some of the same advantages as polyclonal antibody preparations, the methods involved in their production similarly require animal injections. Moreover, polyclonal antibody preparations offer a number of critical advantages over monoclonal antibodies. First, production of useful preparations of polyclonal antibodies takes a shorter period of time than do monoclonal antibodies. Whereas polyclonal antibodies take about 3-4 months to achieve high titer, high affinity reagents, monoclonal antibodies typically take about 6-8 months before useful cell lines are developed and the antibodies are produced in useful quantities. Second, polyclonal antibody preparations typically display higher affinities and higher specificities for their antigen than do monoclonal antibodies. Typical Kd values for polyclonal antibody preparations against peptide antigens is about 1-3 ng/ml, whereas Kd values for monoclonal antibody preparations is about 10-30 ng/ml -- an order of magnitude less

sensitive. Third, the costs of polyclonal antibody preparations are significantly lower than that of monoclonal antibodies. A typical project for production of 1.2 grams costs approximately $820. Costs of an equivalent monoclonal antibody project would cost $3,000-$8,000, depending on the number of clones isolated, and the yields of antibodies required. The lower costs of polyclonal antibody production allow more researchers to take advantage of these important immunological reagents, allowing more studies to be performed at lower costs.

4. **Justification for the Species:** Chickens offer significant advantages over rabbit and other mammals for the production of polyclonal antibodies. First, chickens generally respond better than mammals to highly conserved mammalian gene products. Presumably, this is due to the fact that animals don't respond very well to "self" antigens, because the responding cells have been eliminated from their complement of antibody-producing cells during fetal life. The more differences exist in the primary amino acid sequence of homologous gene products, the higher the probability that the host animal will recognize that gene product as "foreign" and mount a vigorous immune response. If, for example, one injects human fibroblast growth factor type 2 (basic FGF) into a rabbit, the rabbit mounts a modest immune response at best because there are only two amino acid differences between the human and rabbit homologues, and these are conservative serine-to-threonine substitutions. In contrast, there are five amino acid differences between the human and chicken homologues, which include a number of radical substitutions -- e.g., serine-to-cysteine. This makes the chicken homologue of bFGF quite different in structure than that of rabbit bFGF, and chickens are more likely to recognize the human bFGF as a "foreign" antigen.

   A second advantage of chickens over mammals has to do with the fact that the chicken antibody -- the IgY -- lacks an Fc domain involved in complement fixation. This means that chicken antibodies are not recognized by "rheumatoid factors" -- autoimmune IgG's often found in mammals suffering from inflammatory disease. The presence of such "rheumatoid factors," and the fact that such factors often cross react between species, means that mammalian antibodies cannot be used reliably in the detection of gene products in animals with inflammatory disease. In humans, for example, this would abrogate against the use of rabbit antibodies for diagnostic or research purposes when performing biomedical research on a whole host of inflammatory or autoimmune diseases, including rheumatoid arthritis. In addition, the presence of Fc domains on mammalian antibodies means that all cells of the reticuloendothelial system non-specifically bind rabbit antibodies. This is because these cells express Fc receptors. In contrast, chicken antibodies can be used in biomedical studies of the reticuloendothelial system without these problems associated with non-specific binding, since they lack the Fc domain.

   A third advantage of chickens over mammals has to do with the fact that tagged secondary reagents (e.g., fluorescein labeled goat anti-chicken IgY) do not cross-react with various mammalian IgG's. This is an important consideration for double- and triple-labeling studies, where the use of multiple species of antibodies is essential to avoid such cross-reaction problems. Since most antibodies currently on the market are of rabbit, goat, or mouse origins, this makes chicken antibodies a more desirable primary antibody to be

used in conjunction with these more commonly used mammalian antibodies in triple-labeling studies.

A fourth advantage of chickens over mammals as a source of polyclonal antibodies has to do with costs. Since the antibodies are collected from eggs rather than serum, we avoid expensive technician time involved in animal restraint and bleeding. Moreover, the methods used to isolate the IgY antibodies from chicken yolks does not involve the elaborate chromatography steps necessary for isolation of IgG antibodies from mammalian serum, again cutting costs.

A fifth (and related) advantage of chickens over mammals is in the reduction of discomfort for the host animal. The only possible source of discomfort for chickens is the brief restraint used during injections, and the sequelae caused by the adjuvants. In contrast, the level of discomfort to rabbits is likely to be greater because of the lengthy restraint involved in the injections, the anesthesia used during the cardiac puncture procedures for serum collection

5. Justification for the Number of Animals Used in Production of a Given Antigen:

Approximately 85% of our clients use two hens for each antigen, which is the number we recommend for research-scale production of antibodies. This number is a balance between the higher costs of more host animals and the likelihood of producing useful antibodies for the specific applications required.

The reason why one hen is not recommended to clients has to do with the out-bred nature of chickens. That is, each hen can respond quite differently to the same antigen due to differences in their genetic makeup. This is in contrast with inbred stains of mice, in which each animal has the same genetic makeup and is likely to produce qualitatively the same antiserum. It is not uncommon for clients to report that the antibodies purified from one hen are significantly better than those of another hen. Moreover, these differences often relate to their utility in different applications. Anecdotal feedback from clients suggest that, for many antigens, the antibodies from one hen is significantly better for one use (e.g., western blotting) than another hen's in either titers or backgrounds. The use of one hen per antigen, therefore, might have resulted in the production of less-than-satisfactory antibodies, which would have been a waste of time and energy.

It should be noted that some clients (less than 10% of our clients) ask us to inject more than two hens with a given antigen. This is either because the client requires large production of antibodies over a limited period of time, or knows *a priori* that their antigen is a difficult one to make antibodies against. If the reason has to do with bulk production, we work with the client to evaluate their needs. The factors that we consider are:

Each egg contains approximately 100 mg of IgY.
Each hen lays 0.7 eggs per day (this figure takes into consideration molts, etc.).
The proportion of antibody that specifically recognizes the average antigen is 0.8%
(i.e., the proportion of IgY that binds with high affinity on affinity matrices).
The average Kd value of a chicken polyclonal antibody is 2 ng/ml.

From these factors, we can advise a client on how many hens to use for a given project. Of course, the other major consideration to clients is cost, which also tends to limit the numbers of hens used in a given project.

6. **Determination of Pain and Suffering in the Hens:** Although there are no obvious painful or distressful procedures in this protocol, infections at the site of injection or other unforeseen veterinary problems might result in pain and suffering. Consequently, we will use a 3-level system for assessing pain and suffering in the hens, based on easily observable behavioral criteria. This 3-level system will be used to determine whether the hen needs veterinary treatment or possibly euthanasia. It should be noted, however, that it is in the best financial interests of Aves Labs to avoid having the hens experiencing pain and suffering.

   There are, however, two further considerations: First, only ***one*** of the criteria listed below must be met in order to conclude that a hen is within a given category of pain and suffering. Of course, this assessment is more credible if multiple criteria are met. Second, it is understood that hens in higher categories of pain and suffering will likely exhibit behaviors seen with lesser categories, as well. That is, a hen in category B of pain and suffering will likely exhibit behaviors seen in category A, as well as those in category B.

   Level A ("Discomfort"). Birds in Level A of pain and suffering should be monitored daily or every other day, but no intervention need take place.

   1. **Lack of egg production without physiological molting**. Like mammals and other animals, hens that are subjected to stressors, including pain, cease to ovulate. Therefore, egg laying behavior is an early and sensitive indicator of stress, including pain and suffering. However, it should be noted that hens undergoing a physiological molt may also stop egg laying without being in pain. Such a molt, which normally occurs during the autumn months, triggers feather loss and cessation of ovulation, in order to prepare the bird for winter. Other behavioral characteristics need to be considered, therefore, to distinguish between lack of egg production due to pain versus lack of egg production due to molting.
   2. **Repeated scratching or shaking.** Hens in pain will often scratch or shake the affected area to alleviate the painful sensation.
   3. **Lack of vocalizations**. Hens in pain will often cease normal vocalizations and become quite quiet. This is in contrast with normal hens which tend to vocalize frequently.

   Level B ("Moderate Pain"). Birds in Level B of pain and suffering must be monitored daily, and the case should be brought to the attention of Dr. Ciment and/or Dr. Lintner. Whether an veterinary intervention takes place or not will depend on Dr. Lintner's assessment.

   4. **Lack of spontaneous movements, but sitting upright.** Hen in moderate pain will often sit in their cages unwilling to move spontaneously. In order for hens to meet this criterion, they need to be observed over a relatively long period of time (i.e., 10 minutes) and from a distance of at least 10 feet.

Level C ("Severe Pain"). Birds in Level C of pain and suffering must be monitored daily, and the case should be brought to the attention of Dr. Ciment and/or Dr. Lintner. Either of these individuals may determine whether the hen needs to be euthanize to prevent further suffering or not.

5. **Lack of elicited movements**. Hens in severe pain will often remain motionless, even when the observer comes close to the cage or puts his/her hand inside the cage.
6. **Eating and drinking behaviors.** Hens in severe pain will not eat or drink.
7. **Lack of red coloration in the comb.** When a hen is suffering, the comb will often become yellow or pale, in contrast to the comb of a healthy bird, which is bright red.
8. **Drooping eye lids.** Hens in severe pain or suffering will display ptosis or drooping eye lids, in contrast to the eyelids of heathy birds, which are always wide open.
9. **Weakness/Balance.** Hens in severe pain or suffering will appear to be weak and unable to stand. Such hens may have trouble raising from a sitting to a standing position, and may fall over.
10. **Trauma.** Hens with obvious signs of trauma should be treated with antibiotics, as necessary. Such hens should also be assessed carefully for the other criteria associated with Level C pain and suffering.

7. **Unnecessary Duplication of Studies:** In addition to the 6 immune eggs collected from each hen after the injection series is completed, we also collect a second and third batch of 6 immune eggs and store these 12 eggs in the refrigerator for a period of 2 months (after which the yolk membranes begin to break down, rendering the egg useless for IgY purification). If, after receiving the antibodies purified from the first batch of eggs, a client find that one or both hens produce highly desirable antibodies, they can contact us within this period and have us purify additional batches of IgY from these stored eggs. We find that the second batch of 6 eggs yields antibodies with approximately the same titres as the first batch, and that the third batch yields titres that are slightly less, but still useful. In any case, the routine collection and storage of eggs avoids the necessity of having to inject new hens with antigen, or even the necessity of having to perform additional injections into the already-immunized hens. This avoids subjecting hens to unnecessary procedures.

   Another consideration, of course, is that antibodies are consumable reagents, and that they are used up during the course of a set of experiments. For this reason, it may become necessary for the client to ask us to inject additional hens, once their initial supply of antibody becomes low.

8. **Endpoint Criteria:** Most (73%) of our clients ask us to follow our standard protocol, which as described above, involves injecting hens 4 times with antigen, and then collecting 6 immune eggs for production of the IgY fractions. In the standard protocol, the end point is reached when the 6-9 immune eggs are collected (depending on the sizes of the egg yolks). This yields approximately 600 milligrams of IgY per hen. However, as mentioned above, additional batches of immune eggs are collected and stored, in case the client is interested in having us purify additional batches of antibodies.

With the remaining 23% of our clients that ask us to perform additional injections into the hens, the endpoint is determined by the yield of antibody required by the client. In these cases, the client is asked to specify the number of grams of antibody required, and we perform the appropriate number of additional injections and collect the appropriate number of additional batches of eggs using the factors described above.

9. **Euthanasia**: We follow the method recommended method for poultry as described in the American Veterinary Medical Association panel on Euthanasia (2001) -- i.e., cervical dislocation without the prior administration of sedative drugs. In this method, the head is held with one hand with the fingers supporting the base of the occiput, and the legs are held with the other hand. Cervical dislocation is performed by pulling the two hands apart, and feeling for the snap caused by dislocation of the cervical bones. The end point of euthanasia is the lack of a heart beat, as felt under the keel.

10. **Harvesting Organs following Euthanasia:** On rare occasions, a client may ask us to remove organs following euthanasia. For these procedures, the hens will be euthanized as described above, and the endpoint of euthanization will be performed as described above. At that time, the organ(s) of interest will be removed following standard necropsy procedures. Tissues that may be harvested would include the heart, the brain, the spleen, the Bursa of Fabricius, the kidney, the adrenal gland, pectoral muscle.

11. **USDA Categories for Pain and Distress:** Chickens are not covered by the Animal Welfare Act (AWA), and are not subjected to USDA standards. However, the category of Pain and Distress for the hens used in this protocol would be category C -- animals that are subjected to procedures that involve no pain, distress, or procedures that would require the use of pain-relieving drugs.

12. **Personnel Training:** All personnel working with animals are subjected to a set of one-on-one training sessions with Dr. Gary Ciment (Institutional Official, Animal Facility Manager), covering all aspects of animal husbandry, animal handling, standards of custodial maintenance , and the operation of the animal facility physical plant (i.e., water system, electrical system, lighting system, air delivery system, etc.). The new employee is also required to read the Aves Labs Animal Technician Training Manual, which is based on the training manual published by the American Association for Laboratory Animal Science (AALAS), but focuses on the special requirements of hens and the procedures in practice at Aves Labs. This training manual also includes all of the Standard Operating Procedures covering the animal facility and the appropriate treatment and care of the animals in the facility. To determine whether the new employee has successfully acquired the information in this manual, each employee will be required to pass a multiple choice and short answer examination covering this manual.

13. **Facility Inspections:** The facilities are inspected daily by the Animal Care Staff. This inspection requires that the employee examine the food, water system, lights, temperature and relative humidities in both of the buildings. The employee then inspects each hen for general features of health of each hen, including spontaneous movements, the color and texture of the droppings, the fullness of the comb, whether the eyelids are fully open or display ptosis, and egg production. Hens that show features of illness are noted.

Each of the two buildings that make up the animal facilities are also monitored by probes that measure the temperature and relative humidity, as well as power to the building. These measurements are recorded by the Sensaphone 2000 data logging device every 30 minutes of each hour of each day. If any of these parameters go outside a preset range, this device is programmed to automatically dial a set of telephone numbers, including the Aves Labs offices, the homes and cell phones of the Institutional Official and President, and the homes and cell phones of the Animal Care Technicians, notifying them about the out-of-range measurement.

Once a month, the Animal Care Staff also writes up a report of the previous month's animal care status, including hens that died, hens that were euthanized, hens that were noted to be ill, egg production, and other problems that arise. This report is sent to Dr. Marli Linter, the veterinarian, and Dr. Gary Ciment (Institutional Official, Animal Facility Manager) for their review. The purpose of this report is to look for trends in the animal program.

Finally, the facility is inspected by the IACUC every 6 months, in accordance with the rules of the Office for Laboratory Animal Welfare at the National Institutes of Health. These inspections involve Dr. Lintner, Dr. William Woodward (Chair, IACUC), Dr. Ciment, and other members of the IACUC, as their schedules permit.

14. **Justification for the Use of Freund's Adjuvants:** It has been noted that Complete Freund's Adjuvant (CFA) can produce pain and inflammation in rabbits and other mammalian host species often used for antibody production, and that use of CFA can raise the animal's USDA Pain and Distress Category to category D -- animal that are subjected to temporary pain that should be relieve with some sort of analgesic. It should be noted, however, that these studies involved subcutaneous injections and that they were only studied in rabbits.

    At Aves Labs, we have noted that hens with wounds inflicted by other hens, or by an accidental cut will often cease egg production for a couple of weeks until the inflammation has subsided, attesting to the fact that physiologically-stressed hens cease ovulation. We believe, therefore, that egg production is a good indicator of physiological stress. In contrast, we find that the intramuscular injections of CFA that we routinely perform rarely results in the cessation of ovulation. From these observations, we conclude that these differences in species (i.e., rabbits versus chickens) and the route of administration (subcutaneous versus intramuscular) do not cause the same degree of physiological stress suffered by rabbits, and therefore, do not justify the substantially increased costs of alternative adjuvants. This conclusion is, however, not an irreversible one, and will be reconsidered when and if new information emerges that addresses this situation. We will, moreover, monitor hens for the presence of inflammation and areas of necrosis at the sites of muscular injection, as found during necropsies.

15. References

"Working with the IACUC: Writing an Animal Protocol" American Association for Laboratory Animal Science, 2002.

"2000 Report of the AVMA panel on Euthanasia," Journal of the American Veterinary Medical Association 218 (5): 669-696 (2001).