The Influence of Vehicle Gavage on Seasonality of Immune System Parameters in the B6C3F1 Mouse

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Seasonal hypo responsiveness and other immune system variations were observed in female B6C3F1 mice during routine screening tests for immunomodulation. In a retrospective assessment, 4 years of data from over 1200 naive, vehicle, and immunosuppressed (cyclophosphamide-treated) control mice were compiled and analyzed for uniformity and significant circannual pattern of immune response. Endpoints included body, spleen, and thymus weights and an immunotoxicity assessment which enumerates specific antibody plaque-forming cells (PFC) in the spleen following immunization with sheep red blood cells. Dosing vehicles were water, corn oil, or 1% methyl cellulose instilled by oral gavage in a 5–20 ml/kg volume once daily for 5 days. Four days later, terminal organ and body weights were recorded and PFC were quantitated. Upon analysis, individual datapoints were arrayed in consistent circannual and seasonal patterns. In naive mice, the yearly peak response in circannual rhythm (acrophase) for body weight and PFC parameters occurred in the summer, with acrophases for spleen and thymus weights located in the spring. Vehicle gavage modulated the circannual/seasonal means and acrophases of all measured endpoints in distinct patterns which varied by vehicle. Body weight was the endpoint least affected by vehicle treatment. Corn oil was the vehicle resulting in the most dramatic effects on natural rhythm. As expected, the naive mice receiving an ip injection of cyclophosphamide exhibited significant decreases \( p \leq 0.05 \) in circannual mean values for PFC response and relative organ weights when compared to naive controls and the elimination of significant expression of rhythm for PFC parameters. Our results indicate that dosing vehicles alter normal seasonal patterns of biological responses in the mouse. These effects on natural rhythms should be considered in toxicity evaluations, especially when comparing datapoints collected at different times of the year.

Researchers and clinicians are becoming increasingly aware of the importance of chronobiologic considerations in the design of human and animal studies. Each physiologic function of an organism is characterized by a complex time structure of biological rhythms in different frequency ranges which are found at all levels of organization ranging from subcellular particles to cells, tissues, and the intact organism (review by Haus et al., 1983). Circadian variations are perhaps the most commonly observed and studied rhythms. However, other frequency domains are important, including infradian (>)28 hr and including circannual and ultradian (<20 hr) rhythms (Scheving et al., 1994).

Immunologic processes are rhythmic in several frequency ranges and are predictable over time (Fernandes et al., 1977, 1980; Reinberg et al., 1980; Haus et al., 1983). The circannual/seasonal nature of the immune response has been extensively documented for many species, including mouse and man (Bratescu and Teodorescu, 1981; Shifrine et al., 1982; MacMurray et al., 1983; Bureau et al., 1988; Laerum et al., 1988). In both clinical and experimental medicine, the immune response can be selectively stimulated or suppressed by varying the timing or dose in treatment, as susceptibility of the host is also rhythmic (Smolensky and D’Alonzo, 1993).

Immune system parameters of normal female B6C3F1 mice have been studied in our laboratory over a 4-year period during immunotoxicity screening of food flavoring ingredients (Gaworski et al., 1994). These assays typically used one of three common vehicles (water, 1% methyl cellulose, or corn oil) for oral dosing based on each test material’s solubility requirements. Hyporesponsiveness and other endpoint variability were sometimes observed in the plaque-forming cell (PFC) assay, which exhibited a seasonal response when analyzed statistically. The PFC assay measures specific antibody produced following immunization with the T-cell-dependent antigen, sheep red blood cells (SRBC). It provides information about the functional integrity of, and communication among, several cell populations important in antibody-mediated immunity, including T-cells, B-cells, and macrophages. Quantitation of the number of antibody-producing plasma cells, the end result of antigen driven B-
cell differentiation, is a widely used in vitro method for assessing humorally mediated immune function following sensitization in vivo (Abbas et al., 1991). PFC assessment is included in the Food and Drug Administration's directive for evaluation of the immunotoxic potential of direct food additives (Hinton, 1992) and is part of the U.S. National Toxicology Program's tiered approach to immunotoxicology testing (Luster et al., 1992).

Circadian rhythmicity of the PFC assay has been previously reported (Fernandes et al., 1976). Our laboratory has sought to minimize circadian effects by dosing animals, immunizing with SRBC, administering cyclophosphamide, and conducting the PFC assay within a constant circadian stage, consistent with the schedule used during an interlaboratory validation of tests to assess chemical-induced immunotoxicity (Luster et al., 1992).

We have previously reported the seasonal variation in immune response evident in naive B6C3F1 mice over the course of a 1-year period (Ratajczak et al., 1988). We have also documented seasonal differences in antibody formation and host resistance in mice of different strains (Ratajczak et al., 1993). This current investigation is a retrospective analysis of 49 separate immunotoxicity studies conducted over a 4-year period to determine the effects of oral dosing vehicles on the seasonality of various routinely measured immunotoxicity endpoints.

METHODS

Animals and dosing. Female B6C3F1 (C57BL/6 female × C3H male) mice 5 to 6 weeks of age, 17 to 20 g, were obtained from Charles River (Portage, MI) (46 studies) or Harlan Sprague–Dawley (Indianapolis, IN) (three studies). All mice were quarantined for 12 days and randomly housed five-cage in rooms with lights on 0600–1800 hr, lights off 1800–0600 hr. They were provided tap water and pelleted food ad libitum. Animals were weighed and randomized into treatment groups with all groups comparable in pretest body weight (varying ±20% from group mean values).

Within each individual study, one group of 10 animals was given a daily 5-day intragastric injection of one of three vehicles: corn oil, 1% methyl cellulose, or ASTM Type I purified water. Dosing volumes varied from 5 to 10 ml/kg body wt depending on solubility limitations of concurrently dosed test chemicals in separate groups of mice not considered here. (Two studies required a dosing volume of methyl cellulose of 20 ml/kg.) Two additional study groups included undosed (naive) controls and mice with ip-injected cyclophosphamide as positive assay controls for immunosuppression, which did not receive vehicle treatment and were not sham gavaged. Altogether, a total of 1211 mice used as controls in 49 separate immunotoxicity studies are the focus of this retrospective analysis. A distribution of animals by vehicle group and season of treatment is shown in Table 1.

Plaque-forming cell assay. The PFC response to SRBC was evaluated according to a modification (Thomas et al., 1985) of the Jerne plaque assay using Cunningham chambers (Jerne et al., 1963; Cunningham and Steenber, 1968). Briefly, all mice were injected ip with 2 x 10⁷ SRBC (Colorado Serum Co., Denver, CO) 4 days prior to assay and after 5 days of vehicle treatment. Positive control animals were injected ip with 80 mg/kg cyclophosphamide in Dulbecco's phosphate-buffered saline, 24 hr prior to assay.

### TABLE 1

<table>
<thead>
<tr>
<th>Seasonal Distribution of Immunotoxicity Studies</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
<th>Fall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive control</td>
<td>50 (5)</td>
<td>89 (9)</td>
<td>170 (17)</td>
<td>178 (18)</td>
</tr>
<tr>
<td>Corn oil</td>
<td>30 (3)</td>
<td>30 (3)</td>
<td>99 (10)</td>
<td>67 (7)</td>
</tr>
<tr>
<td>Methyl cellulose</td>
<td>10 (1)</td>
<td>60 (6)</td>
<td>30 (3)</td>
<td>37 (4)</td>
</tr>
<tr>
<td>Water</td>
<td>10 (1)</td>
<td>0 (0)</td>
<td>40 (4)</td>
<td>67 (7)</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>25 (5)</td>
<td>45 (9)</td>
<td>85 (17)</td>
<td>89 (18)</td>
</tr>
</tbody>
</table>

* Number of female B6C3F1 mice. (Number of studies performed is given in parentheses.)

Four days postimmunization, mice were killed by cervical dislocation for aseptic collection of target organs. Single spleen cell suspensions were prepared by mechanical dissociation and suspended in complete medium (RPMI 1640 culture medium (Bio-Whittaker, Walkersville, MD) supplemented with 50 units/μg/ml penicillin/streptomycin (P/S), 50 μg/ml gentamicin, 200 μM l-glutamine, 25 mM Hepes buffer, and 2% heat-treated (56°C, 30 min) fetal bovine serum). Viability was assessed by trypan blue exclusion.

A mixture of equal volumes of 80% guinea pig complement (fresh frozen guinea pig serum) and 16% SRBC (v/v) was made, kept on ice, and diluted with medium (RPMI plus P/S). Equal volumes of diluted spleen cells and SRBC/guinea pig complement mixture were added to duplicate Cunningham PFC chambers, sealed with petroleum jelly, and incubated at a 37°C humidified atmosphere for 1 hr. The resulting IgM anti-SRBC plaques were counted with the aid of a plaque viewer (Belloco Biotechnology, Vineland, NJ).

Collection of weight data. Body, spleen, and thymus weights were collected from all animals at the time of PFC assay.

Data evaluation. Each data set was analyzed for time effect across four 3-month seasons (using December 22 as start of winter) and across 12 equal "monthly" intervals (30.5 days each, beginning January 1) by analysis of variance (ANOVA). Monthly data are not shown (except for a visual depiction in Fig. 1) due to lack of studies in all months for each vehicle. A significant difference (p < 0.05) indicated a time effect between the seasonal means within each treatment group. By season, the mean value of each treatment group and its corresponding naive control was analyzed for statistically significant (p < 0.05) using ANOVA followed by Dunnett's test where appropriate (SigmaStat Software, Version 2.0, SPSS, Inc., Chicago, IL).

When evaluating the seasonal means, presence of a significant time effect across the seasons did not necessarily mean that a circannual rhythm had been detected. Circannual rhythmicity was investigated by the fit of a 12-month or 12 + 6-month cosine to each variable by the least-squares (single amplitude test) and was considered significant if p < 0.05. Mammalian organisms appear to experience an annual cycle (circannual rhythm) that is under circadian clock control (Luster et al., 1992). Circannual rhythm characteristics were obtained from the 12-month component of the multiple cosine model, since it generally resulted in a better fit for all series. As seen in Fig. 1, the rhythm characteristics estimated by the single cosine method include:

- **MESOR** Middle-estimating statistics of rhythm; middle value of the fitted cosine representing a rhythm-adjusted mean (differs from the arithmetic mean if data are not equidistant).
- **Amplitude** Difference between the minimum or maximum point of the fitted cosine curve and the MESOR (i.e., one-half of the peak-trough distance; one-half of the total predictable change in rhythm).
- **Acrophase** Time of peak value in a fitted cosine function.

A rhythm was detected if the amplitude differed from zero (the nonzero amplitude test) and was considered significant if p < 0.05.
FIG. 1. An example of circannual rhythm calculated by the single cosinor method using the individual body weight data from naive female B6C3F1 mice collected over a 4-year period. Data grouped by “month” and season are depicted here to show fit to the 1-year cosine curve.

Since the serially independent data were collected at unequal intervals over a number of years, the single cosinor method was selected to provide the best objective estimate of both the circannual amplitude and the acrophase (Klemfuss and Clopton, 1993), especially important for analyzing studies with seasons containing few or no datapoints, such as for the vehicle water. Circannual means (MESORs) were compared between naive controls and each experimental group using the Bingham test of rhythm parameters (Bingham et al., 1982).

RESULTS

Seasonal Means

Seasonal means were calculated for each individual data series compiled over 4 years (Figs. 2–4), with endpoints including body, relative spleen and thymus weights, PFC/spleen, and PFC/10^6 viable spleen cells. While mean values for methyl cellulose and water in the winter and/or spring are included for comparison, note that these groups contained less than three studies (<30 total mice) each.

Body weight. Terminal body weight data of female B6C3F1 mice expressed as seasonal means are shown in Fig. 2. Body weights of naive mice demonstrated a significant time effect across the four seasons. Similarly, animals receiving vehicles by oral gavage or ip-injected cyclophosphamide also exhibited this effect (although not statistically significant for water due to the lack of spring data), with peak responses occurring in the spring and summer. When comparing the means of each vehicle to the naive control, by season, the only significant difference was seen in methyl cellulose dosed in the summer. Generally, seasonal means for body weight were lowest in the fall and winter. Of the measured immunotoxicity endpoints, body weight was affected the least by cyclophosphamide treatment or by oral gavage of dosing vehicles.

Relative spleen and thymus weights. The seasonal means for spleen and thymus weights of female B6C3F1 mice are presented as body weight-relative organ weights in Fig. 3. As seen with body weights, the organ weights of naive mice demonstrated a significant time effect across the seasons, with peak responses occurring in the spring. For relative spleen and thymus weights, corn oil significantly suppressed the normal seasonal pattern observed in naive animals in spring, summer, and fall, essentially equalizing the response between these three seasons. Ip-injected cyclophosphamide
Vehicle Gavage Modulates Murine Immunity

Consistently in naive control mice for each endpoint, with acrophases (rhythmic peaks) for PFC responses and body weight located in the summer; acrophases were observed in the spring for relative spleen and thymus weights.

When compared to naive mice, vehicle gavage modulated the circannual means (MESORs) and acrophases of the immune response parameters in distinct patterns which varied by vehicle, although only minimal effects of vehicle gavage were seen in body weight. Corn oil significantly reduced MESOR values for both relative target organ weights and PFC/spleen. A circannual rhythm was detected for body weight and PFC/spleen, with peaks in the summer, and for thymus weight, with a peak in late winter, but the circannual pattern for relative spleen weight was lost. As expected, the immunosuppressant cyclophosphamide dramatically reduced MESORs for all measured PFC and organ weight values when compared to naive controls, but circannual rhythms were present for body and organ weights with acrophases comparable to naive controls.

**Discussion**

Evaluation of body weights, organ weights, and PFC assay results measured during immunotoxicity screening tests in

**Circannual Rhythms**

Table 2 presents circannual rhythm parameters calculated by the least-squares (single cosinor) method using all individual datapoints. A circannual rhythm ($p \leq 0.05$) occurred drastically reduced each seasonal mean for both relative target organ weights, eliminating the seasonal time effect for relative spleen weight. A significant time effect was observed in relative thymus weight for each study group, with highest values in winter or spring.

**PFC Values.** Seasonal means for PFC/spleen (total organ) and PFC/10$^6$ viable spleen cells activities are shown in Fig. 4. A significant time effect across the seasons was seen for both naive and vehicle-treated animals. The lowest PFC response of each treatment group was evident in the winter, where significant reductions from naive control levels occurred in mice gavaged with methyl cellulose and water. In the summer, PFC levels were significantly lower in mice gavaged with methyl cellulose and corn oil. Cyclophosphamide treatment 24 hr prior to terminal sacrifice caused drastic suppression in PFC activity.

**Fig. 3.** Seasonal means ± SE for 4 years of individual body weight-relative spleen and thymus weight data of female B6C3F1 mice. No mean value is available for water in the spring. CY, cyclophosphamide; Methyl-C, methyl cellulose. *Significant for time effect (TE) of treatment across four seasons (ANOVA: $p \leq 0.05$). **Significantly different from naive control (Dunnett's test: $p \leq 0.05$).

**Fig. 4.** Seasonal means ± SE for PFC enumeration from 4 years of individual data in the female B6C3F1 mouse, expressed as PFC/spleen and PFC/10$^6$ viable spleen cells. No mean value is available for water in the spring. CY, cyclophosphamide; Methyl-C, methyl cellulose. *Significant for time effect (TE) of treatment across four seasons (ANOVA: $p \leq 0.05$). **Significantly different from naive control (Dunnett's test: $p \leq 0.05$).
control B6C3F1 mice over a 4-year period demonstrated a yearly rhythm for all parameters. These data serve to reinforce the importance of considering (and recording) the time of year in which toxicity testing is conducted (Sothern and Gruber, 1994). Interestingly, not all parameters displayed the same rhythmic pattern. Acrophases for body weight and PFC responses occurred in the summer, while rhythmic peaks for spleen and thymus weights were observed in the spring.

The selection of a vehicle for oral administration of test articles in toxicity screening is often based solely upon the ability of that vehicle to suspend or solubilize the active ingredient, while neglecting the capacity of the vehicle to alter normal physiologic response. Our results demonstrate that oral gavage of vehicles in mice modulates the natural circannual rhythm for all parameters. These data serve to reinforce the importance of considering the time of year in which toxicity testing is conducted (Sothern and Gruber, 1994). Interestingly, not all parameters displayed the same rhythmic pattern. Acrophases for body weight and PFC responses occurred in the summer, while rhythmic peaks for spleen and thymus weights were observed in the spring.

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endpoints in concurrent studies in B6C3F1 and CD1 mouse strains given the same diet (identical lot numbers of rodent chow) revealed consistent, although different, seasons of peak response, implicating genetic control of rhythm (Ratajczak et al., 1993). This observation minimizes diet as a confounding influence on rhythm changes in our studies. To the extent possible, we reduced dietary confounders in our studies by using one feed supplier and brand of rodent chow, although specific lot numbers varied over the course of 4 years. The same constraint to a single vendor and product number was applied to the procurement of the corn oil and methyl cellulose used as vehicles.

Stress is caused by changes in homeostasis and hormone modulation, including secretion of catecholamines, adrenocorticotropic, and corticosteroids (Vogel, 1987; Matamoros and Levine, 1996). Different alterations in immune function can result from acute or chronic changes in this neurochemical environment where cells of the immune system exist and function (Monjan, 1981; MacMurray et al., 1983). Hormonal modulation of the immune response is influenced by animal housing conditions, including variation in the number of animals housed per cage (Grewal et al., 1997) and presence of the opposite sex. Our study animals were grouped in a consistent manner for both quarantine and study periods. Males were not present in the housing areas at any time, which could have influenced cyclicity of estrous (Bingel, 1972). Circannual variation in serum corticosterone levels has also been reported in mice bred in-house under strict handling, lighting, and housing conditions (Haus and Halberg, 1970), evidence for seasonal variation despite attempts to control for external factors intrinsic to animal experimentation.

In addition, the stress resulting from oral gavage may cause changes in an animal’s biological response (Roberts et al., 1995). Unfortunately, any contribution of a bolus oral dose of vehicle cannot be distinguished in our studies from the possible effects resulting from the oral gavage procedure, since our naive and cyclophosphamide-injected animals were not sham gavaged.

Biological rhythms are not unique to the immune system; cyclic modulation is naturally inherent in human and animal models (Haus et al., 1983). Despite a number of possible confounding factors, these biological rhythms may be altered in a reproducible, although as yet unexplained, manner. These changes may not be readily apparent upon analysis of individual acute studies, but may become evident during long-term investigations, especially when chronic dosing regimens are employed. Therefore, it is important to have an appreciation of an animal’s natural rhythm of response and any alteration in this normal response caused by vehicle treatment.

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