Modeling and Predicting Immunological Effects of Chemical Stressors: Characterization of a Quantitative Biomarker for Immunological Changes Caused by Atrazine and Ethanol

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Previous studies demonstrate that the effects of one chemical stressor on selected immunological parameters can be predicted on the basis of the area under the corticosterone concentration vs. time curve. However, it is not clear if this is applicable to other chemical stressors. The present study was conducted to determine if the stress-induced immunological effects of atrazine and ethanol could be predicted, and if it is feasible to use one immunological parameter as a biomarker of stress to predict the quantity of changes expected in other immunological parameters. The area under the corticosterone concentration-versus-time curve (AUC) was measured in mice treated with ethanol (EtOH, 4, 5, 6, or 7 g/kg by oral gavage) or atrazine (ATZ, 100, 200, or 300 mg/kg, ip). The effects of the same dosages of these chemicals on thymus and spleen cellularity, lymphocyte subpopulations in the thymus and spleen, expression of MHC class II protein on splenocytes, antibody responses to keyhole limpet hemocyanin, and natural killer cell activity were determined. Models were derived describing the relationship between corticosterone AUC and immunological changes induced by these chemicals. The results for these chemical stressors were more similar to results obtained from mice subjected to restraint stress than from mice treated with exogenous corticosterone. Some effects were greater than predicted on the basis of the stress response alone, indicating other mechanisms of immunotoxicity. One of the parameters (MHC class II expression) was evaluated as a predictive biomarker for stress-related immunosuppression, and the results suggest it could be suitable for that purpose.

Key Words: stress, corticosterone, atrazine, ethanol, predictive model, biomarker.

Drugs and chemicals frequently induce a physiological stress response in rodents, especially when administered at the highest dosages, in routine safety evaluations. Seventeen compounds representing a wide range of chemical categories were cited as examples in a previous publication (Pruett et al., 1999), and recent reports provide confirmatory evidence for some of these (Lall and Dan, 1999; Zabrodskii and Germanchuk, 2000). Other recent studies provide evidence that methylendioxymethamphetamine (ecstasy) (Pacifici et al., 2000), trifluralin (Rawlings et al., 1998), ethyl carbamate (Cha et al., 2001), and cocaine (Stanulis et al., 1997) can induce stress responses. In addition, there is convincing evidence that immunosuppression induced by the synergistic action of lipopolysaccharide and vomitoxin is mediated at least in part by the stress response to these compounds (Islam et al., 2002). Stress responses are also very likely to be associated with the therapeutic use of cytokines known to activate the hypothalamic-pituitary-adrenal axis (e.g., TNF-α, IL-6, or IL-1) (Turnbull and Rivier, 1999). Immunotoxicity assessment is now mandated by the U.S. Environmental Protection Agency (EPA), Office of Prevention, Pesticides, and Toxic Substances Guidelines 870.7800, as part of the new-product registration process. The U.S. Food and Drug Administration has issued a similar draft Guidance document outlining immunotoxicity evaluation procedures for new drugs. The EPA Guidelines recommend a high dosage near the maximum tolerated dose for immunotoxicity testing in rodents. At dosages this high, it is likely that many of the compounds tested will cause a stress response.

The effects of stress responses on the immune system are complex, but relatively intense responses that persist for a few hours or more generally cause immunosuppression (Pruett, 2001). Because corticosterone is one of the major immunosuppressive mediators of stress responses, it was selected as a mediator that might predict the quantity of immunosuppression caused by stress responses. This does not imply that corticosterone is the only stress-induced mediator that can affect the immune system, but that it will likely increase in magnitude in parallel with other mediators and thus serve as a useful surrogate for the intensity of the overall stress response. Quantitative assessment of the increased exposure to corticosterone requires determination of the area under the corticosterone concentration-versus-time curve (AUC) for the entire period during which corticosterone levels are elevated (Pruett et al., 1999). It was anticipated that the immunological effects of exogenous corticosterone would be less than the effects of a full stress response...
An immunological parameter that could serve as a biomarker of stress would be useful in routine safety assessments. If stress-induced changes in such a parameter correlated to other parameters (e.g., spleen and thymus cellularity or the antibody response to a T-dependent antigen), this pattern of changes could serve as a useful indicator of the role of stress in chemical-induced immunosuppression. Some immune parameters that are affected by stress are either already recommended or could easily be included in them. A predictive biomarker approach would seem preferable to experiments requiring separate sets of animals in which pharmacological agents were used to block stress mediators to determine the degree to which observed immunological effects of chemicals are caused by these mediators.

MATERIALS AND METHODS

Mice. Female C57BL/6 × C3H F1 (B6C3F1) mice were used in this study. These mice were obtained through the National Cancer Institute’s animal program. B6C3F1 mice were selected because of their use in immunotoxicology studies sponsored by the National Toxicology Program and in our previous studies in which exogenous corticosterone, restraint stress, and propanil were evaluated (Pruett and Fan, 2000; Pruett et al., 1999, 2000a,b). Mice were used in experiments at 8 to 12 weeks of age, following a two-week period during which they were allowed to recover from shipping stress. Mice were maintained on a 12-h light/dark cycle (lights on at 7:00 A.M., off at 7:00 P.M.), and they were given food (Harlan Tek-Lad 22/5 rodent diet) and water ad libitum. Mice were specifically pathogen-free, and the animal facility is routinely monitored for mouse pathogens and adventitious agents, using sentinel animals. No sentinels were positive during the period of these studies. Animal care and use were conducted in accord with the NIH Guide and the regulations of LSU Health Sciences Center in a facility that is accredited by the American Association for Accreditation of Laboratory Animal Care.

Administration of ethanol (EtOH) and atrazine (ATZ). ATZ was obtained from ChemService (West Chester, PA). It was administered intraperitoneally at dosages of 100-, 200-, or 300-mg/kg body weight in food-grade corn oil. In one experiment, ATZ was administered by oral gavage in corn oil at 500 mg/kg to match the high dosage used in the National Toxicology Program’s study (as described on the NTP web site at http://ntp-server.nih.gov/docs/pub-IT0.html). EtOH was administered by oral gavage at dosages of 4, 5, 6, or 7 g/kg using a 32% solution in tissue culture-grade water. These dosages produce peak blood EtOH levels ranging from ~0.2 to 0.5% (Carson and Pruett, 1996). Previous studies demonstrated that the corticosterone response to vehicle administration subsides within 1–4 h. This yields a negligible corticosterone AUC value, which only serves to add an additional data point near the 0 value for corticosterone AUC to any models developed (Pruett et al., 1999). Therefore, naive control groups, not vehicle control groups, were used for these modeling studies.

Determination of corticosterone AUC values for EtOH and ATZ-treated mice. Six identical sets of mice were treated with ATZ at 0 (naive), 100, 200, or 300 mg/kg at 9:00 P.M. Thus, there were 4 groups of mice in each set and there were 5 mice in each group. One of these sets of mice was bled by decapitation at 1, 2, 4, 6, 8, and 12 h after ATZ administration. A similar approach was used for EtOH, with dosages of 0 (naive), 4, 5, 6, or 7 g/kg. Mice were bled one cage at a time in a separate room to prevent the induction of stress responses in the remaining mice. Serum was separated from each blood sample and the corticosterone was quantified using a radioimmunoassay kit (DPC, Los Angeles, CA) (Pruett et al., 1999). Graphs of the data were produced using DeltaGraph software (SPSS Inc., Chicago, Ill.), and the area between the naive control values and the treated values (AUC) was determined as described previously using NIH Image software (Pruett et al., 1999).

Measurement of immunological parameters. Immunological parameters were evaluated by routine methods exactly as described in our previous studies (Han and Pruett, 1995; Pruett et al., 1999, 2000a; Weiss et al., 1996; Wu et al., 1994). The methods are summarized in following paragraphs. The group size was 5 in all experiments. Cell suspensions were prepared by pressing the spleen or thymus between the frosted ends of glass microscope slides, and spleen and thymus cellularity were determined using an electronic cell counter (Coulter model Z1, Coulter Electronics, Hialeah, FL). Previous studies indicated that the greatest changes in spleen and thymus cellularity and in the lymphoid subpopulations in these organs occurred 24 h after the initiation of the stressor (Pruett et al., 2000a), whereas the greatest changes in NK cell activity and major histocompatibility complex (MHC) class II expression occurred 12 h after the initiation of the stressor (Pruett et al., 1999). Therefore, these times were used in the present study.

Labeling for flow cytometry was conducted using fluorescent-labeled antibodies specific for CD4, CD8, B220 (CD45R), and MHC class II (I-Aαβγδε) using antibodies from BD Pharmingen (San Diego, CA). Cells were labeled in V-bottom microtiter plates at a concentration of 107 cells per well, and an isotype control was used for each antibody. Gates were set using the unlabeled control cell and the isotype controls so that 2% or less of the cells labeled with the isotype control was used for each antibody. Cells were labeled for 30 min on ice and then washed twice in FACS buffer (1% bovine serum albumin in PBS). Cells were resuspended in 500 μl of FACS buffer and analyzed using a FACSCalibur (Becton-Dickinson and Company, San Jose, CA). Lymphocytes were identified using PI exclusion. Cells were gated on the lymphocyte population and analyzed using FlowJo software (Tree Star, Ashland, OR). A minimum of 10,000 events were collected per experiment for each antibody.

Measurement of NK cell activity. Splenic NK cell activity was measured 12 h after initiation of treatment, using a standard 4 h 51Cr release assay with YAC-1 target cells (Pruett et al., 1999). Effectors (spleen cell) to target cell ratios of 100:1, 50:1, and 25:1 were used, and values were expressed as lytic units per 107 splenocytes (Bryant et al., 1992) to provide a single value for modeling.

Mice were immunized with 100 μg/mouse keyhole limpet hemocyanin KHL (Pierce Chemical Co.) 12 h after treatment with EtOH or ATZ, as in our previous studies (Pruett and Fan 2000; Pruett et al., 2000b). Two weeks after immunization mice were bled under methoxyflurane anesthesia. This is the time at which near maximum antibody levels occur (Kruszewska et al., 1995), and it is the time used in our previous studies. Serum was diluted 1:25, 1:50, and 1:100, and IgG1 and IgG2a antibodies specific for KHL were measured by ELISA just as described in our previous studies (Pruett and Fan, 2000; Pruett et al., 2000b). The results indicated linear relationships of the antibody titration curves, so the absorbance values from the ELISA at the 1:25 antibody dilution were normalized (by dividing by the mean value of the naive control group and multiplying by 100), and these normalized values were used for subsequent regression analyses. Control serum from nonimmunized mice was included in every assay and yielded absorbance values less than 0.06 (as compared to typical values of 0.3–0.4 in sera from immunized mice).
Statistical and modeling methods. In most cases, linear models best described the data obtained. The runs test was done for every linear model, and none of the models in the present study had a significant nonlinear component. In addition, linear models were used because they facilitated comparison of results between different experiments. Linear regression was performed using the Prism software package (GraphPad, San Diego, CA), as described previously (Prue et al., 1999). The 95% confidence intervals were calculated by the program and are shown on the graphs presented here, which were generated using Prism. Statistical comparison of pairs of lines was done using Prism, which implements the method described by Zar (1984). This method determines if the slope or the elevation (or Y-intercept) differ significantly between any pair of lines. To compare values at particular points on different lines, the 83.7% confidence intervals were calculated using StatView software (SPSS, Chicago, IL). Contrary to the common assumption, failure of 95% confidence intervals of two values to overlap does not indicate that the values are different at the 0.05 probability level. In fact, this indicates an even lower p value. Failure of the 83.7% confidence intervals to overlap indicates that the values are significantly different at the p ≤ 0.05 level (Barr, 1969; Nelson, 1989).

The significance of differences between control and treatment groups was determined by analysis of variance (ANOVA) followed by Dunnett’s test, in some experiments.

RESULTS

Determination of Corticosterone AUC Values for ATZ and EtOH

The peak corticosterone response to ATZ occurred at 6 h (Fig. 1), and it was greater than noted for EtOH (Fig. 2). In fact, the peak value for ATZ of ~1200 ng/ml is among the highest value we have observed for any stressor. The corticosterone AUC values are shown in Figure 1, and they are dose-responsive, as expected. The evaluation of ATZ sponsored by the National Toxicology Program utilized oral gavage to administer ATZ, and the highest dosage was 500 mg/kg (the study is described on the NTP Web site at http://ntp-server.niehs.nih.gov/htdocs/pub-IT0.html). To determine whether the highest dosage and the route of administration (which were different from those used in the present study) also increased endogenous corticosterone, five mice were treated with ATZ by gavage (in corn oil), and five mice were treated with vehicle. Three h after dosing, the mice were bled, and the following corticosterone values were measured: vehicle control, 164 ± 88; ATZ 676 ± 79. This indicates that the route of administration of ATZ used in the present study (ip) is not unique in the induction of a stress response by this chemical.

The peak corticosterone level in EtOH-treated mice was observed 1 h after dosing (Fig. 2), and at ~800 ng/ml it was somewhat less than reported in our previous study (~1100 ng/ml) (Carson and Pruett, 1996). However, it should be noted that in the previous study, EtOH was administered in the morning, whereas in this study EtOH was administered at 9:00 pm. It is well established that some of the physiological effects of EtOH in mice vary considerably when EtOH is administered at different times of day (Deimling and Schnell, 1980). At all dosages of EtOH, corticosterone values had returned to baseline by 12 h. The values for corticosterone AUC are shown in Figure 2, and these values are dose-responsive as expected.

In a previous study, it was determined that handling and injection of vehicle induce a small increase in corticosterone levels (AUC, 303 ng/ml-h) (Pruett et al., 1999), but this was not associated with significant changes in any of the immunological parameters measured. Therefore, only naive control groups were used in this study.

Linear Models of Corticosterone AUC vs. Thymus- and Spleen-Cell Numbers and Subpopulation Percentages in EtOH- and ATZ-Treated Mice

Previously published data (Pruett et al., 2000a) from mice treated with exogenous corticosterone or restraint are shown for comparison along with data from mice treated with ATZ or EtOH in the present study (Figs. 3 and 4). The slopes of the regression lines were significantly different for ATZ and EtOH with regard to CD4⁺/CD8⁺ cells in the thymus, with ATZ causing a greater decrease in this subpopulation than EtOH (at equivalent corticosterone AUC values). Our previous study
demonstrated that the difference in slope between the line for exogenous corticosterone and the line for restraint is also significant, with exogenous corticosterone causing a greater decrease than restraint (Pruett et al., 2000a). The regression lines for restraint and EtOH were not significantly different, whereas the lines for restraint and ATZ were significantly different. The slope was more negative for ATZ than for restraint, indicating that ATZ has a greater effect than restraint (at equivalent corticosterone AUC values). Together these results indicate that the effects of ATZ and corticosterone on the percentage of CD4⁺CD8⁺ cells in the thymus were minimal, and the slopes for these two treatments were not significantly different (Figs. 3 and 4). Thus, the increased percentages that were noted for some subpopulations do not reflect increases in the total percentage of CD4⁺CD8⁻ cells in mice treated with corticosterone or ATZ. EtOH and restraint had little effect on this parameter, as indicated by slopes that were small and were not significantly different from each other (Fig. 4). Similar patterns were noted for CD4⁺CD8⁻ cells in the thymus (Figs. 3 and 4), except that the increases in slope for ATZ and corticosterone were less than noted for CD4⁺CD8⁻ cells. The effects of EtOH and ATZ on the percentage of CD4⁺CD8⁻ cells in the thymus were minimal, and the slopes for these two treatments were not significantly different (Figs. 3 and 4). It should be noted that all treatments caused a decrease in the total number of thymocytes (Figs. 3 and 4). Thus, the increased percentages that were noted for some subpopulations do not reflect increases in the total number of thymocytes.
number of these cells, but simply indicate that CD4\(^+\)/CD8\(^+\) cells are decreased to a greater extent than other cell types by some of the treatments. Because these cells are the dominant subpopulation in the thymus, even a relatively small decrease in this population mathematically leads to a substantial increase in the percentage of other subpopulations.

The number of nucleated cells in the spleen was decreased dose-responsively by ATZ, but only slightly by EtOH (Figs. 5 and 6). The slopes of the lines described by these results were significantly different. The cellular subpopulations analyzed were affected by ATZ, but not significantly by EtOH (as indicated by ANOVA). The percentage of B cells (B220\(^-\)) was substantially decreased by ATZ, producing a strongly negative slope for the percentage of B220\(^-\) cells vs. corticosterone AUC. The other treatments caused only very small decreases, even at the highest corticosterone AUC values. As expected, the percentage of CD4\(^-\) and CD8\(^-\) T cells increased in a manner that corresponds to the decrease in B-cell percentage in response to ATZ, but not EtOH (Figs. 5 and 6).

Linear Models of Corticosterone AUC versus Expression of MHC Class II Protein on Splenocytes in Mice Treated with ATZ and EtOH

As shown in Figure 7, ATZ and EtOH dose-responsively decreased the expression of MHC class II molecules on splenocytes. The data shown were normalized (by defining the naive control group as 100%) using the natural logarithm of the fraction of cells that expresses the highest level of MHC class II proteins. This represents approximately 20% of total splenocytes or half of the B lymphocytes (Pruett et al., 1999). Expressing the data this way provides a better indication of changes in MHC II than reporting the total number of MHC II positive cells, because most cells do not completely lose MHC II expression in response to stress, but express lower levels (Weiss et al., 1996). The results were expressed as the natural log to produce a linear relationship that did not exhibit a significant nonlinear component (as determined by the runs
The regression lines for ATZ and EtOH were significantly different, but the differences were small. In fact, as we have reported previously for exogenous corticosterone, restraint, and propanil (Pruett et al., 2000b), all treatments evaluated to date have yielded very similar linear relationships.

**Linear Models of Corticosterone AUC vs. NK Cell Activity in Mice Treated with ATZ and EtOH**

Data shown in Figure 8 indicate that ATZ and EtOH dose-responsively decrease splenic NK cell activity. The slope for NK cell lytic units vs. corticosterone AUC was greater for EtOH than any other treatment examined in this study or previous studies (Pruett et al., 2000b). In fact, comparison of all combinations of these lines indicates that the slope of the line for EtOH is significantly different from the slopes of each of the other lines, whereas, the other lines are not significantly different from each other.

**Linear Models of Corticosterone AUC vs. IgG1 and IgG2a Antibody Responses to KLH in Mice Treated with ATZ and EtOH**

ATZ and ETOH dose-responsively decreased the IgG1 and IgG2a responses to KLH (Fig. 9). Regression lines obtained in a previous study (Pruett and Fan, 2000) using mice treated with exogenous corticosterone or restraint are shown for comparison.

The IgG1 response to KLH was affected similarly by restraint, ATZ, and EtOH at equivalent corticosterone AUC values. None of the slopes of these lines were significantly different from each other, and the elevations of the ATZ and EtOH lines were not significantly different from the elevation of the restraint line.
However, exogenous corticosterone had a smaller effect on the IgG1 response to KLH than to any of the other treatments (the elevation for the exogenous corticosterone line was significantly different from the elevation of all the other lines).

The IgG2a response to KLH was affected equally by ATZ and EtOH at equivalent corticosterone AUC values. The effect of these compounds was more similar to the effects of restraint than of exogenous corticosterone (Fig. 8). However, there was a small but significant difference in the elevation of the lines for ATZ and ETOH when compared to restraint. Both compounds were slightly more suppressive than restraint.

Overall Correlation between Observed Values for Immunological Parameters in Mice Treated with Chemical Stressors and Values Predicted Using Linear Models Derived from Mice Treated with Restraint or Exogenous Corticosterone

The data shown in Figure 10 represent a comparison of the ability of the linear models obtained using restraint and exogenous corticosterone to predict each of the immunological parameters evaluated in this study for ATZ and EtOH, as well as the same parameters evaluated in a previous study in mice treated with propanil (Pruett et al., 2000b). The observed value of each parameter at an ATZ dosage of 200 mg/kg, an EtOH dosage of 7 g/kg, and a propanil dosage of 100 mg/kg (which yielded similar corticosterone AUC values of 3387, 4068, and 4383, respectively) was compared to the values predicted using the corticosterone AUC and the restraint or the corticosterone linear model. The parameters used in this analysis were: MHC class II expression, NK cell activity, IgG1 and IgG2a responses to KLH, percentage of major subpopulations in the thymus (CD4+, CD8+, CD4+CD8+, CD4+CD8-, CD4-CD8+), percentage of major subpopulations in the spleen (B220, CD4+, CD8+), thymus cellularity, and spleen cellularity. The results demonstrate correlation between observed and predicted values for both the restraint and the corticosterone models (Fig. 10).
However, the correlation coefficient was substantially higher for the restraint models (0.66) than for the corticosterone models (0.39). Also shown in Figure 10 are the idealized lines for perfect correlation. Both of the actual lines are above the ideal lines, as a result of clusters of data points for which the predicted value was greater than the observed value. Interestingly, there were very few points below the ideal line, which would indicate predicted values lower than observed values. Overall, this indicates that the chemicals tested suppress some of the parameters examined to a greater extent than can be accounted for by stress alone, but the effects of the chemicals on essentially all parameters were at least as great as predicted by the stress models (corticosterone or restraint). In general, restraint had greater effects than corticosterone alone and thus was more predictive of the effects of chemicals.

The Use of MHC Class II as a Predictive Biomarker for the Immunological Effects of Chemical Stressors

The results in Figure 10 suggest that the correlation between predicted and observed values for immunological parameters, using the restraint models, may be sufficient and that restraint models could be used to predict the immunological outcome of chemical stressors. In addition, MHC class II expression has consistently been the most sensitive parameter in our studies with regard to suppression by stressors. All chemical stressors tested to date, as well as exogenous corticosterone and restraint, produce similar linear relationships between corticosterone AUC and MHC class II expression. We have previously presented evidence using complementary experimental approaches indicating that endogenous corticosterone is almost completely responsible for the suppression of MHC class II expression in EtOH-treated mice (Weiss et al., 1996). Thus, MHC class II expression would seem to be a reasonable candidate for consideration as a biomarker for chemical-induced stress. As an initial step to determine the feasibility of this idea, the values predicted for selected parameters using linear models derived from mice treated with restraint are shown in Table 1 (with the associated 83.7% confidence intervals). Also shown are the values determined using the regression lines obtained in mice treated with ATZ, EtOH, or propanil. The single corticosterone AUC value at which restraint suppressed MHC class II expression by 50% was used in all these calculations. It was used as the “x” value in the equation for each line to calculate the “y” value (immunological param-

<p>| TABLE 1 |
| Prediction of Immunological Effects of Stress Using Corticosterone AUC Values |</p>
<table>
<thead>
<tr>
<th></th>
<th>ATZ</th>
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<th>Propanil</th>
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<td>Observed</td>
<td>Observed</td>
<td>Observed</td>
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<td>70 (55–84)</td>
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<td>IgG2a response</td>
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<td>73 (68–78)</td>
<td>69 (60–74)*</td>
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<td>76 (64–87)</td>
<td>95 (86–105)</td>
</tr>
<tr>
<td>Thymus cellularity</td>
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<td>58 (47–71)*</td>
<td>81 (69–93)</td>
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<tr>
<td>MHC II expression</td>
<td>50 (47–54)</td>
<td>54 (51–59)</td>
<td>40 (35–45)*</td>
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</table>

*Observed values significantly different from the predicted value.

Note. Predicted values were derived from linear models from restraint stress experiments. Numbers represent % of naive control value.
eter value normalized to the naive control value as 100%). Table 1 illustrates the results of these calculations. Values whose 83.7 confidence intervals do not overlap are significantly different at the p < 0.05 level (Barr, 1969; Nelson, 1989), and these values are indicated in table Notes. These results indicate that the linear models derived from corticosterone AUC values in mice subjected to restraint stress provide reasonably good predictions of chemical-induced immunological effects (at equivalent corticosterone AUC values). As expected, there were a few cases in which the effect of the chemical was greater than the effect predicted on the basis of the stress response alone. This was particularly notable in the case of EtOH. Because different chemicals affected thymus subpopulations differently, and spleen subpopulations were not substantially affected by any treatment, these parameters were not used.

Although the predictive value of the restraint models is good when a reference corticosterone AUC value is used as the point of comparison, the utility of a biomarker approach would be greater if the extra time and animals required to determine the corticosterone AUC for each chemical were not required. If, for example, the dosage of chemical at which MHC class II is suppressed to 50% of the naive control value could be used as a reference point, it would be possible to use this value and the linear regression models derived from the dose-response relationship for each immunological parameter, to determine the effect of each chemical at this reference dosage on each immunological end point. To determine if this gives similar estimates of immunological effects to the corticosterone AUC values, the effect of each chemical at the dosage required to suppress MHC class II expression by 50% for each immunological end point was compared to the value predicted by restraint models using the corticosterone AUC value yielding 50% suppression of MHC class II. The results of this analysis are shown in Table 2, and they demonstrate that the prediction of the effects of all three stressors is actually slightly better by this approach than by using corticosterone AUC values (as in Table 1). Data shown here for propanil were calculated using previously published results (Pruett and Fan, 2000; Pruett et al., 2000b). These results demonstrate that the minimum (the stress-induced portion) of the immunological effects of three chemically disparate stressors could be effectively predicted on the basis of a single immunological parameter (MHC class II expression) using only the linear models that describe the effects of restraint stress on these parameters. Once these restraint models are established, chemical dosages, instead of corticosterone AUC values, can be used to predict effects on several immunological parameters, based only on the effects of that chemical on MHC class II expression.

## DISCUSSION

The results reported here indicate that linear models can be derived that allow prediction of several immunological parameters, including those that are recommended by EPA and FDA for inclusion in immunotoxicological safety evaluation (NK lytic activity and the primary humoral response to a T-dependent antigen). If evaluation of MHC class II expression was also measured, the effects of the drug or chemical on each of these parameters could be compared to the values predicted using the restraint models derived here. Close agreement with the expected values would suggest that the observed effects are mediated primarily by stress. In fact, substantial suppression of MHC class II per se would be sufficient to at least suggest stress as a possible cause of immunosuppression. Few stimuli other than stressors (Celada et al., 1993; Zwilling et al., 1990, 1993) have been described that downregulate basal MHC class II expression (which is primarily on B cells in the spleen). No drugs or chemicals have been reported to suppress basal MHC class II expression, except by inducing a stress response. Of course, this parameter has not been routinely evaluated in immunotoxicology studies, so more data are needed to determine how reliable an indicator of stress-induced immunosuppression MHC class II expression alone will be. As we have noted previously (Pruett et al., 1999), these modeling studies are not intended to predict immunotoxicity on the basis of corticosterone AUC values alone, because direct effects of chemicals on the immune system (in addition to stress-mediated effects) are always possible. However, comparing observed values and values expected on the basis of stress effects could provide a useful estimate of the extent to which particular immune changes are stress mediated.

EtOH was selected for these studies on the basis of numerous reports demonstrating that it activates the hypothalamic-pituitary-adrenal axis in rodents and humans (Mendelson and Stein, 1966; Pruett et al., 1998). Atrazine was selected because it is the most abundantly used conventional agricultural chemical in the U.S. (according the U.S. EPA Web site at http://www.epa.gov/oppbead1/pestsales/97pestsales/table8.htm), and

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### TABLE 2

**Potential Use of MHCII as a Predictive Biomarker for Stress**

<table>
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<th>Predicted</th>
<th>Observed</th>
<th>Observed</th>
<th>Observed</th>
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<tr>
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<td>81 (76–86)</td>
<td>70 (64–76)</td>
<td>74 (67–81)</td>
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<tr>
<td>MHC II expression</td>
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<td>50 (46–54)</td>
<td>50 (42–60)</td>
<td>50 (45–55)</td>
</tr>
</tbody>
</table>

*Note.* Values for immunological effects of the chemicals were calculated from the dose-response line for each chemical at the dosage yielding 50% suppression of MHC class II. These are compared to the values predicted using corticosterone AUC values induced by restraint at the AUC value yielding 50% suppression of MHC class II. Numbers represent % of naive control value.

*Observed values significantly different from the predicted value.*
an immunotoxicology assessment by the National Toxicology Program indicates that a high dosage causes approximately 50% loss of thymocytes (these results are summarized on the NTP Web site at http://ntp-server.niehs.nih.gov/htdocs/IT-studies/IMM94002.html), a parameter that is particularly sensitive to glucocorticoids. We previously evaluated propanil in an identical set of experiments, because it has been reported that propanil induces a vigorous stress response that is responsible for at least some of its immunological effects (Cuff et al., 1996). The prevalence of the induction of stress responses by immunotoxic drugs or chemicals is not known. However, there are at least 21 drugs or chemicals that have been reported to induce a stress response (listed in Pruett et al., 1999). In addition, we have reported that sodium methyldithiocarbamate (the third most abundantly used agricultural pesticide in the U.S. induces a stress response, and that this is completely responsible for the thymic atrophy observed in mice treated with this compound (Myers and Pruett, 2001). Because the EPA recommends the use of a high dosage, near the maximum tolerated dosage (which by definition produces some signs of overt toxicity), it seems likely that, at least at the higher dosages, stress responses will be common in immunotoxicology safety evaluation. Although the serum corticosterone AUC values for EtOH and ATZ were linearly related to the dose of each compound (Figs. 1 and 2), it is clear that the effects of ATZ on serum corticosterone are complex. For example, corticosterone is maximal at 1 h for both the 100 and 200 mg/kg dosages, but the maximum level occurs at 6 h for the 300-mg/kg dosage. It is not clear what effects such differences may have on immune parameters, but it is likely that the differences, as well as time of day (in relation to normal circadian changes in corticosterone), have some impact on immunological outcomes.

The results reported here are applicable only to acute effects of a single dose of chemical stressor. Because immunotoxicological safety evaluations often involve dosing daily for 28 days, it will be important to determine if these relationships persist after 28 days of dosing. It is possible that some degree of tolerance to the stress-inducing effects of some drugs or chemicals will develop over 28 days. However, it seems unlikely that this will occur for all chemicals and drugs. Although it is also possible that the immune system will become tolerant to the effects of stress-induced neuroendocrine mediators, it is unlikely this will be of sufficient magnitude to substantially alter all immunological effects. For example, it is well documented that chronic stress and metabolic disorders such as Cushing’s syndrome, that involve increased concentration of stress hormones, cause chronic changes in the immune system (Kronfol et al., 1996; Vedhara et al., 1999).

In addition to the implications of these findings with regard to safety evaluations, there are implications with regard to the effects of stress in general and drug and chemical-induced stress in particular. The effects of the chemical stressors are more similar to the effects of restraint than to the effects of exogenous corticosterone. This was expected, because it was assumed that immunosuppressive stress mediators in addition to corticosterone would be induced by restraint and that this would lead to greater immunosuppression. This was observed for most parameters, and greater than expected suppression was noted for NK cell activity in mice treated with EtOH and for the percentage of B cells in the spleen with ATZ. This may be due to direct action of these chemicals. Alternatively, it is possible that corticosterone is regulated differently and affects the immune system differently when it is given exogenously (presumably representing only the afferent limb of the stress response) than when it is produced endogenously (representing efferent and afferent limbs of the stress response). We initially chose to evaluate IgG1 and IgG2a instead of IgM because of reports that stress differentially affected these two immunoglobulins (Pruett and Fan, 2000). However, we did not observe meaningful differences in this study or in our previous study (Pruett and Fan, 2000).

The effects on lymphocyte subpopulations in the thymus were actually reversed, suggesting the possibility that mediators other than corticosterone might actually protect CD4⁺CD8⁺ thymocytes from the action of corticosterone. The effects of EtOH on thymocyte subpopulations were generally intermediate between the effects of stress and exogenous corticosterone, whereas the effects of ATZ were more similar to the effects of restraint (Figs. 3 and 4). Results for mice treated with propanil were also similar to results for mice treated with restraint (Pruett et al., 2000b). Because changes in thymic subpopulations are obviously under complex regulation and are not consistent between restraint and exogenous corticosterone or among the chemicals tested, this parameter was not included in the predictive models shown in this study.

The observation that a single stress response induced by EtOH or ATZ, lasting no more than 8 h, can cause substantial changes in several immunological parameters is interesting and has not previously been reported. The effect of EtOH on NK cell activity and thymus cell number and subpopulations has been reported previously (Han and Pruett, 1995; Han et al., 1993; Weiss et al., 1996; Wu and Pruett, 1996b), but the effects of atrazine and the other effects of acute EtOH have not been previously reported in the peer-reviewed literature. The dosages of EtOH used in this study produce blood EtOH levels that are relevant with regard to at least some human binge drinkers (Carson and Pruett, 1996), and high blood EtOH levels are associated with a stress response in humans (Pruett et al., 1998). Thus, the models derived here have potential for application in predicting immunological changes in human alcoholics or binge drinkers. Although the dosages of ATZ used here are higher than typical for environmental exposure of humans, they are representative of dosages that would be required to meet the testing requirements of the EPA for approval of a new pesticide, which indicates that the highest dose should be near the maximum tolerated dose (U.S. EPA,
Decreased spleen and thymus weight, preferential loss of splenic B cells, and decreased resistance to B16F10 melanoma cells have been noted the day after 14 daily doses of ATZ at 500 mg/kg (by gavage) (http://ntp-server.niehs.nih.gov/htdocs/IT-studies/IMM94002.html). However, there was no effect on the primary IgM response to sheep erythrocytes. This study was conducted through the National Toxicology Program. The results are available online, but they have not been published.

In any case, the effects reported were mostly similar to those noted in the present study. The difference with regard to effects on a primary antibody response could reflect differences in the antigens used or the immunoglobulin isotypes measured. It is also possible that differences could be attributable to the use of multiple doses in the NTP study, possibly allowing tolerance to develop to some effects. An earlier study did not identify any meaningful, dose-related immunosuppression using a single dose of ATZ at 27.5–875 mg/kg (Fournier et al., 1992). However, immunological parameters were not evaluated until 7 days after dosing. This probably was a sufficient time to allow recovery of most immunological parameters to normal values. In any case, it should be noted that oral gavage of ATZ at the high dosage used in the NTP-sponsored evaluation of this compound (500 mg/kg) significantly increased serum corticosterone in the present study. This suggests that the stress response induced by ATZ is not entirely dependent on the route of administration and was likely involved in the immunosuppression reported in the NTP study.

The ultimate goal of this research is to complete a risk-assessment parallelogram that would allow extrapolation of immunotoxicology data obtained in mice to humans. By obtaining data similar to that described here using mouse blood (rather than spleen) as a source of immune system cells and by treating human subjects with cortisol and obtaining similar immunological data from them, it should be possible to directly relate stress-mediated immunotoxicity in mice and humans. This could then be taken further by measuring host resistance in mice under that same condition, and using these data and the relationships between immunological parameters in mice and humans to estimate the effects of stressors on host resistance in humans.

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REFERENCES


Kruszewska, B., Felten, S. Y., and Moynihan, J. A. (1995). Alterations in psychomotor activity and immune parameters in mice under that same condition, and using these data and the relationships between immunological parameters in mice and humans to estimate the effects of stressors on host resistance in humans.

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REFERENCES


drinking: Role of Ro15–4513-sensitive gamma aminobutyric acid receptors, tolerance, and relevance to humans. Life Sci. 63, 1137–1146.


