PHARMACOGENETIC STUDIES ON THE DRUG-RELATED LUPUS SYNDROME

Differences in Antinuclear Antibody Development and Drug-Induced DNA Damage in Rapid and Slow Acetylator Animal Models

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Pharmacogenetic study of an inbred mouse model system derived from A/J (slow acetylator) and C57BL/6J (rapid acetylator) parental strains shows that spontaneous occurrence of antinuclear antibodies is associated with the slow acetylator phenotype although the development of spontaneous and procainamide-induced antinuclear antibodies is a dissociable process. In another study using primary cultures of intact hepatocytes obtained from slow and rapid acetylator rabbits, observations indicate that the amount of DNA damage induced by exposure to hydrazine and arylamine containing foreign compounds depends on the concentration of the foreign compound used as well as on the acetylator phenotype. Exposure to hydralazine induced greater DNA damage in slow acetylator hepatocytes whereas exposure to the arylamine carcinogen, 2-aminofluorene, induced greater DNA damage in rapid acetylator hepatocytes.

A lupus erythematosus-like syndrome is sometimes seen in patients receiving therapeutic doses of hydralazine, procainamide (PA), or isoniazid (1). Each of these drugs contains either a hydrazine or an arylamine group which undergoes N-acetylation, an important early step in their elimination from the body (2–6). Persons with a low capacity for N-acetylation, such as phenotypic slow acetylators, are more likely to develop drug-induced lupus or some manifestations of this disorder from these drugs than rapid acetylators (7). Slow acetylators may also be found in abnormally high numbers in certain groups of patients with spontaneous lupus erythematosus (8). These correlations have suggested more than a simple pharmacologic relation between drug-induced lupus and the slow acetylator phenotype and we have devised new experimental approaches to seek such possibilities.

We have found from pharmacogenetic studies in an inbred mouse model system that spontaneous occurrence of antinuclear antibodies (ANA) is associated with the slow acetylator phenotype (9,10), although the development of spontaneous and procainamide-induced ANA is a dissociable process. Also, we have found that the amount of drug-induced DNA damage in primary cultures of intact hepatocytes obtained from rapid and slow acetylator rabbits is dependent on the specific hydrazine drug or arylamine to which the hepatocytes are exposed as well as the acetylator phenotype.

Acetylator phenotype and ANA

Twenty inbred strains of mice, including NZB, NZW, and NZB/W F_1 female hybrids, were screened for their ability to N-acetylate arylamine substrates. The NZB/W F_1 female hybrid mice are characterized by spontaneous appearance of ANA and several other complications of human systemic lupus erythematosus (11). One parental strain, the NZB, is characterized by a severe Coombs positive hemolytic anemia while the other parent, NZW, is apparently normal immunologically. Although the autoimmune disease of these mice is not worsened by lupus-inducing drugs (12,13), the disease of another autoimmune strain, A/J, is exacerbated by these agents (14–16).

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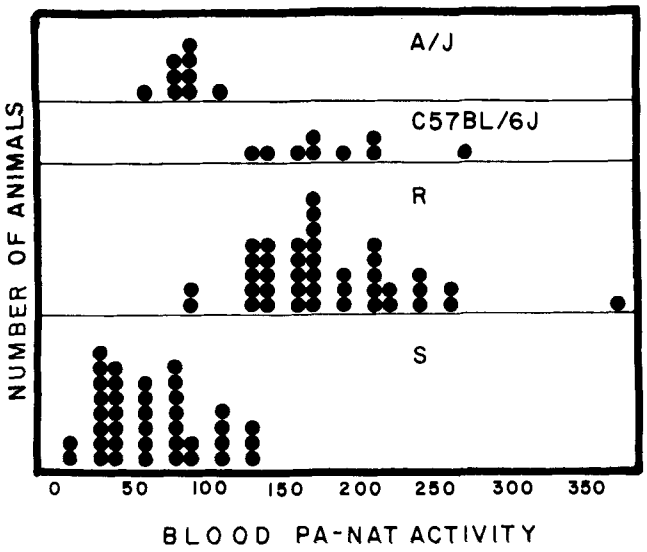

Figure 1. Blood PA-NAT activity in rapid and slow acetylators (pmol/min/mg protein). C57BL/6J and A/J mice can be distinguished on the basis of PA as well as PABA acetylation. Offspring in the F_2 and backcross populations which were phenotyped as rapid or slow acetylators of PABA are also appropriately rapid and slow acetylators of PA. Correlation coefficient of blood PA-NAT versus PABA-NAT activity in F_1, F_2, and backcross mice was 0.77 ($P < 0.005$). Each symbol represents 1 animal. (From J Pharmacol Exp Ther 213:485–490, 1980. Reproduced by permission.)

The principal substrates employed for screening were p-aminobenzoic acid and sulfamethazine since they show unimodal and bimodal populations of acetylation capacity respectively in human and rabbit populations (17–19). In this survery, we found that the A/J strain is a slow acetylator phenotype (20). A/J mice can be differentiated from C57BL/6J mice, a rapid acetylator autoimmune resistant inbred strain selected for these characteristics, by several measures of acetylation capacity: 1) by little or no detectable N-acetyltransferase activities for p-aminobenzoic acid, procainamide (10), aminofluorene, and benzidine in blood (21); 2) by the excretion of less urinary acetylsulfamethazine after oral administration of sulfamethazine (11.4 ± 4.4% for A/J versus 28.3 ± 5.5% for C57BL/6J) (10); and 3) by an 8–10 fold difference in N-acetyltransferase activity for aminofluorene or benzidine in liver (21).

Genetic analysis of distributions for F_1, F_2, and *backcross* progeny for both liver and blood reveals inheritance patterns consistent with segregation at a single locus of 2 major codominant autosomal alleles for aminofluorene N-acetyltransferase activity. This genetic hypothesis is further supported from analysis of recombinant inbred lines developed from A/J and C57BL/6J strains (22).

These observations suggested that A/J mice could be a useful model for studying the human lupus diathesis since this strain is characterized by a predisposition to spontaneous and drug-induced ANA and by the slow acetylator phenotype. To evaluate the importance of the slow acetylator phenotype in the lupus diathesis, A/J mice were mated with C57BL/6J mice and associations were sought between slow acetylator phenotype and both spontaneous and procainamide-induced ANA among offspring from F_1, F_2, and backcross matings.

Since procainamide was the provocative drug in this study, it was important to demonstrate that metabolism of procainamide reflects the acetylator polymorphism in mice. We showed that in both parental strains and animals in the F_2 and backcross offspring slow or rapid p-aminobenzoic acid acetylators were also slow or rapid acetylators of procainamide (Figure 1). However, no appreciable differences in either procainamide acetylation in vitro by liver and certain other tissues or by procainamide acetylation in vivo was apparent between the parental strains.

Mice 3 months of age were provided procainamide in the drinking water at a concentration sufficient to supply 20 mg/kg/day. ANA determinations were made in each mouse throughout the 37 weeks of the study of untreated and procainamide-treated mice as an indication of the autoimmune process occurring either spontaneously or as a result of procainamide administration. An indirect slide test for ANA was em-

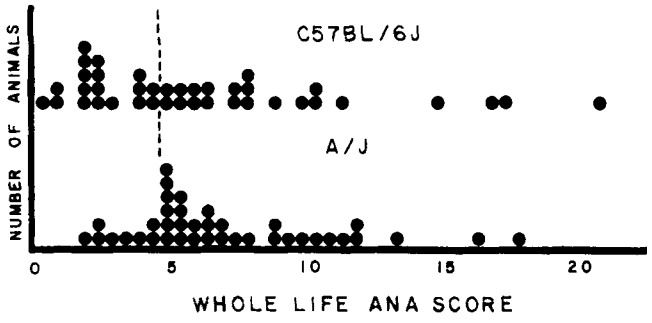

Figure 2. Whole-life ANA scores of parental type animals. Whole-life ANA score is the sum of 8 determinations per mouse during the 37 weeks of the experiments. Individual tests were graded by using the scale previously described (10). A/J mice (both sexes) had higher whole-life ANA scores than C57BL/6J mice ($\chi^2 = 5.4$; $\chi^2 > 3.8$ is significant, $P < 0.05$). (From J Pharmacol Exp Ther 213:485–490, 1980. Reproduced by permission.)

ployed which utilizes a peroxidase-conjugated rabbit antimouse IgG to visualize ANA deposited in the nuclear substrate (23).

By using this test we confirmed the findings of Ten Veen and Feltkamp (14) that A/J mice have a higher incidence of ANA-positive sera spontaneously than C57BL/6J mice (Figure 2). It was also noted that ANA responsiveness is probably a genetic trait since backcrosses with the A/J parent produced offspring with greater ANA positivity than offspring from backcrosses with C57BL/6J parents (Figure 3). Whole-life ANA scores, the sum of 8 ANA determinations in each mouse, also support the difference in ANA positivity between strains (Figure 4). It is also evident from the whole-life ANA scores in the F_2 generation that an association still occurs after two generations between slow acetylator phenotype and high titers of spontaneous ANA.

Effects of procainamide exposure on whole-life scores can be seen in Figure 5. Data in Figure 5 from procainamide-treated mice (open-circles) show a shift to the right indicating that ANA levels are induced in A/J mice by procainamide as Ten Veen and Feltkamp (14) observed. Contrary to their observations, however, we found that procainamide significantly *suppressed* ANA titers in C57BL/6J mice as shown by the shift of the open circles to the left. Phenotypic slow acetylator offspring, unlike their slow acetylator (A/J) parents, showed procainamide-suppression of ANA scores, while ANA titers in rapid acetylator offspring were

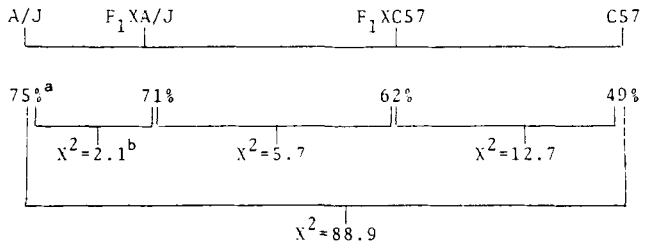

Figure 3. ANA responsiveness in parental lines and their backcrosses. **a,** Average percentage of positive ANA tests for all animals of a given type; 8 determinations per animal. Backcross animals were pooled on the basis of negative findings for sex and drug treatment. **b,** χ^2 values are computed on the basis of the average percentage of positive ANA reactions and the total number of determinations (from 320 to 636 per group). $\chi^2 > 3.8$ is significant, $P < 0.05$. (From J Pharmacol Exp Ther 213:485–490, 1980. Reproduced by permission.)

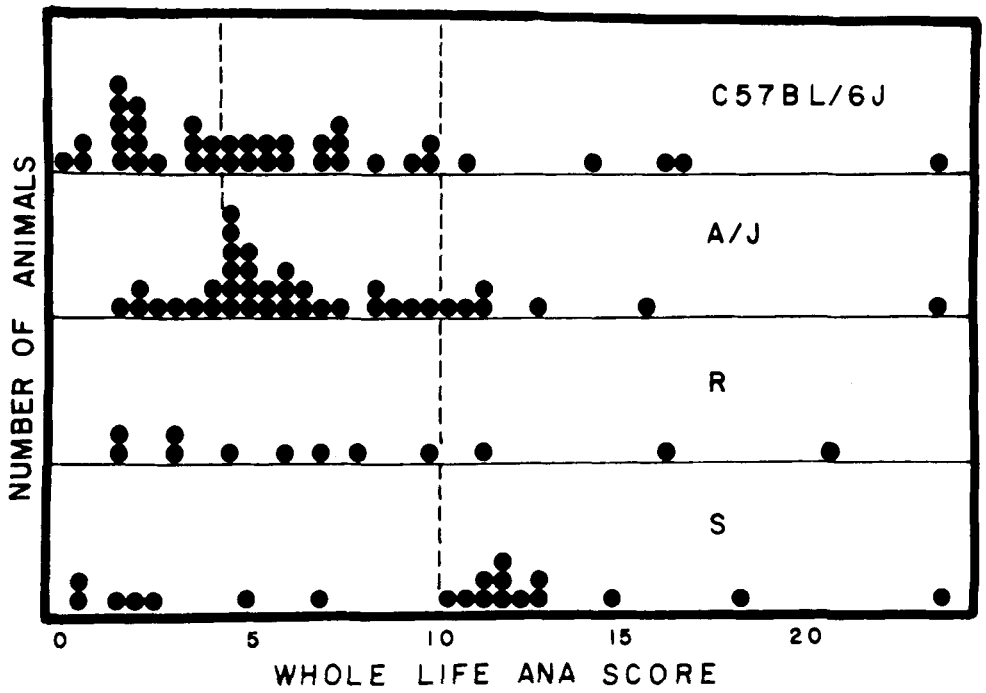

Figure 4. Whole-life spontaneous ANA scores in rapid and slow acetylator mice. High spontaneous ANA scores were associated with the S phenotype as found in the A/J parental strain. Phenotypic S mice had significantly higher whole-life spontaneous ANA scores than R ($\chi^2 = 4.80$), A/J ($\chi^2 = 13.1$), or C57BL/6J mice ($\chi^2 = 5.4$). $\chi^2 > 3.8$ is significant, $P < 0.05$. Each symbol represents 1 animal. (From J Pharmacol Exp Ther 213:485–490, 1980. Reproduced by permission.)

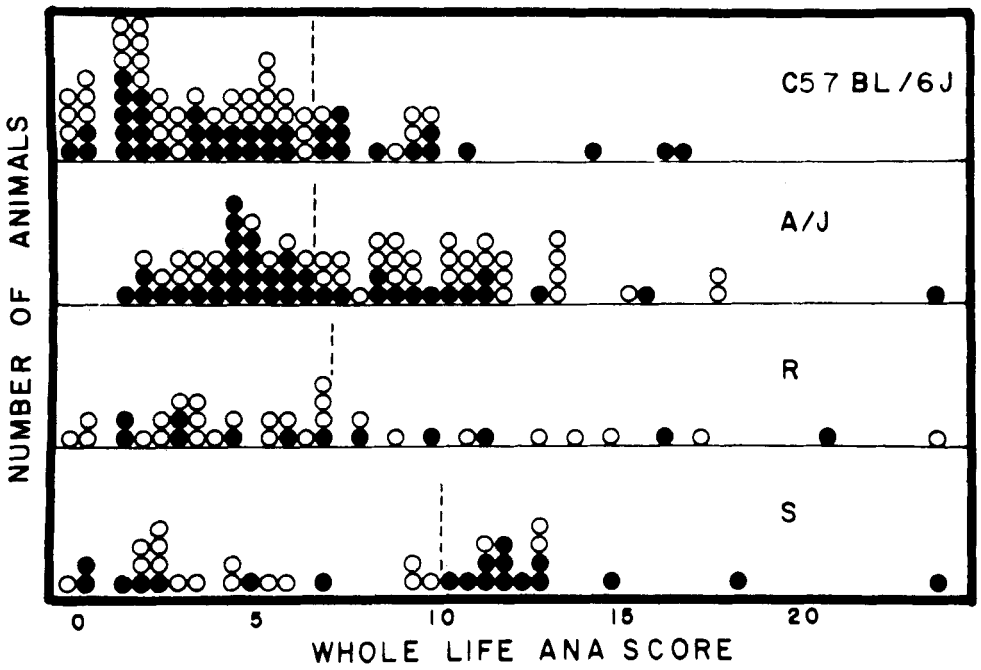

Figure 5. Effect of PA administration on whole-life ANA scores. Chronic PA administration (O) elevated ANA scores in A/J mice ($\chi^2 = 10.7$) and depressed scores in C57BL/6J mice ($\chi^2 = 5.6$). Phenotypic S mice, unlike their slow acetylator parents (A/J), showed PA suppression of ANA scores ($\chi^2 = 9.1$) indicating the genetic independence of acetylator phenotype from PA-inducible ANA. PA had no effect on ANA scores of R mice. $\chi^2 > 3.8$ is significant, $P < 0.05$. Each symbol represents 1 animal. (From J Pharmacol Exp Ther 213:485–490, 1980. Reproduced by permission.)

unaffected by exposure to procainamide. These findings suggest that spontaneous and procainamide-induced ANA in mice are dissociable processes and that the slow acetylator phenotype is associated with spontaneous occurrence of ANA, perhaps as a genetic marker. On the other hand, a possible causal relationship is not excluded even though such a relationship is not apparent from these studies.

The unexpected occurrence of ANA suppression in C57BL/6J mice and slow acetylator F_2 offspring derived from C57BL/6J parents raises some interesting questions about the mechanisms by which procainamide alters ANA titers. Although the slow acetylator phenotype is a liability in human drug-induced lupus, this seems not to be the case in mice. Perhaps slow acetylator phenotypes do not have elevated levels of procainamide or have such high *spontaneous* ANA titers that they cannot be induced further. Of interest in connection with the data we report are the observations that the severe autoimmune state of NZB was not worsened by the lupus-inducing drugs either (12,13).

Procainamide-induced ANA suppression also raises the possibility that procainamide itself might be cross reactive with nuclear antigens and thus block ANA reactions in mice having the drug in their serum. Moreover, such cross reactivity, should it be demonstrable, might be the means by which procainamide induces ANA. This hypothesis of cross reactivity seems unlikely, however, since none of the sera from control or procainamide-treated mice had anti-procainamide antibodies irrespective of ANA titers, nor did procainamide added directly to ANA-positive mouse serum pools alter their titer.

We have suggested a more speculative mechanism for drug-induced ANA (10) i.e., that drugs capable of inducing lupus have a distinct pharmacologic property which enables them to act directly on the immune system either in unchanged form or through metabolites and that the ability of the immune system to respond to this property is subject to genetic variability which appears to covary with the slow acetylator trait. In support of such a possibility it has been proposed that loss of a population of suppressor T cells accompanies autoimmune susceptibility in mice (24,25) and that replenishment of these cells can prevent or reverse some autoimmune phenomena (26). Perhaps this population of cells is "weak" in some strains and "strong" in others and the lupus-inducing drugs being toxic to or suppressive of

these cells, affect the weak strains more readily. (See addendum.)

Dependence of DNA damage on acetylator phenotype and substrate specificity

Tissue disorders that characterize drug-induced lupus are believed to be mediated by antibodies to nucleic acids, nucleoproteins, or both (27,28) but very little appears to be known about the mechanism by which these antibodies are produced. Should adducts be formed between lupus-inducing drugs (or their metabolites) and nuclear components, this could either alter the DNA of recipient cells to increase its antigenicity or alter the nuclear components of recipient immune systems to induce immune nonresponsiveness. Either of these mechanisms might account for the effects seen in drug-induced lupus.

Formation of adducts between a lupus-inducing drug and nuclear components might occur directly (29) although a great deal of evidence has been accumulated during recent years to indicate that the toxicologic actions of many injurious chemicals including procainamide (30) involve metabolic activation ultimately resulting in combination between reactive metabolites of the chemical and macromolecules of the host tissues. A propos of such reactions involving lupus-inducing drugs, Lorand et al (31) have observed that hydralazine and isoniazid are excellent substrates for plasma transglutaminase and are incorporated into serum proteins. Recently Buxman (32) has extended this work to include intracellular epidermal transglutaminase by showing that albumin and nucleoproteins are excellent acceptors of these drugs and that the drug-albumin and drug-histone conjugates formed are highly antigenic, eliciting drug-specific antibodies in immunized rabbits. These investigations are relevant to the problem of drug-induced lupus although they do not account for the role of the acetylator phenotype as a possible etiologic factor in the production of the antibodies associated with this disorder.

Recently our laboratory has been interested to discern whether there is a correlation between the genetic differences in individual N-acetylating capacity of arylamines such as aminofluorene and differences in nucleic acid adduct formation (33). Enzymatic activation of aminofluorene is a complex process involving N-acetylation, N-hydroxylation, and esterification. Two steps in this process, separated by N-hydroxylation, involve N-acetyl transfer: 1) the CoASAc-dependent formation of amides via N-acetyltransferase, and 2) the CoASAc-independent generation of reactive N-acetoxyarylamines from arylhydroxamic acids via arylhydroxamic acid N,O-acetyltransferase. The latter enzyme removes the N-acetyl group of the hydroxamic acid and produces a reactive species capable of forming arylamine-nucleic acid and protein adducts (34).

Comparison of the properties of this acetyltransferase with those of the CoASAc-dependent N-acetyltransferase revealed a number of similarities. Subsequently we showed that 1) both activities were high in rapid acetylator rabbits and low in slow acetylator rabbits; 2) they could not be resolved by various protein purification techniques which exploit differences in solubility, charge, and size; and 3) they both migrated as a single symmetrical protein band on polyacrylamide gel electrophoresis. Thus it appears that the enzyme responsible for arylamine-nucleic acid adduct formation and the genetically polymorphic N-acetyltransferase are properties of the same enzyme in rabbit liver.

These findings suggested that hepatocytes from rapid and slow acetylator rabbits might be useful in determining whether DNA damage induced by substances such as hydralazine and 2-aminofluorene is dependent on acetylator phenotype. A number of short-term tests have been devised to evaluate the potential of specific chemicals for genotoxic effects (35). One of these, the hepatocyte primary culture (HPC/DNA) repair test, assesses chemically induced DNA damage by measuring ^{3}H-thymidine incorporation by autoradiography during DNA repair in freshly isolated hepatocytes in monolayer cell culture. Intact hepatocytes were isolated from rabbits of both acetylator phenotypes by collagenase perfusion and DNA damage was assessed. Previous studies had shown that the genetic N-acetyltransferase polymorphism seen in intact rabbits and liver homogenates (18) was also seen in the isolated hepatocytes (36). Preliminary results from an experiment in which hepatocytes were exposed to a range of hydralazine concentrations (10^{-5}–$10^{-2}M$) showed that the amount of DNA damage depends on the hydralazine concentration to which cells are exposed. In the same experiment unscheduled DNA synthesis was seen in the *slow* acetylator hepatocytes exposed to $10^{-3}M$ hydralazine but not in rapid acetylator hepatocytes exposed to $5 \times 10^{-3}M$ hydralazine. (HPC/DNA repair test was carried out by Dr. C. A. McQueen and Dr. G. M. Williams of the Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, New York.) This suggests that slow acetylation augments the genotoxic effect of hydralazine.

In a similar experiment employing various con-

centrations of 2-aminofluorene, unscheduled DNA synthesis occurred in *rapid* acetylator hepatocytes exposed to $10^{-4}M$ 2-aminofluorene while it was barely detected in slow acetylator hepatocytes at that concentration of the arylamine. Thus, rapid acetylator hepatocytes were more sensitive to 2-aminofluorene-induced genotoxicity than slow acetylator hepatocytes. Additionally, it is evident that DNA damage in rapid and slow acetylator rabbit hepatocytes depends on the acetylator phenotype for both of the compounds tested, although the degree of DNA damage resulting from exposure to hydralazine is greater in slow acetylator hepatocytes while the opposite appears to be the case for 2-aminofluorene.

Evidently differences in chemical structure must be regarded as critical determinants of DNA damage under the conditions of this test. Although this conclusion is not entirely unexpected, we believe that this is the first demonstration of a difference in DNA damage related to the acetylator phenotype difference. The mechanism by which the observed differences were generated is not established although it is reasonable to expect their explanation may lie in differences in the metabolism of hydralazine and 2-aminofluorene and possibly in reactive metabolites derived from them. Such considerations should also take account of differences in reactivity of nucleic acid components and other macromolecules for induced metabolites. It may be pertinent in connection with this possibility that hydrazine, another agent that has been reported to induce a lupus-like state (37), specifically reacts with pyrimidine bases of DNA but not with purine bases (38,39). 2-Aminofluorene-nucleic acid adducts, on the other hand, are formed through purine (guanosine) bases (34).

We conclude that acetylator phenotype can affect adduct formation between nucleic acids and lupus-inducing drugs such as hydralazine and that such a mechanism may occur in human beings and partially account for the predisposition of slow acetylators to hydralazine-induced lupus.

Addendum

Evidence for a cellular rather than an antigen-based mechanism has recently been obtained. Serum from patients treated with PA suppressed Con A mitogenesis in cultures of peripheral blood mononuclear cells, but serum from N-acetylprocainamide-treated patients did not. This effect could not be seen by adding either drug to normal serum. Conversion of a greater portion of procainamide to its acetylated metabolite could thereby confer a degree of protection from the PA-induced inhibitory factor. The nature of this inhibitory factor is not known. However, it is significant that serum from SLE patients also specifically inhibits Con A mitogenesis. This common feature of spontaneous and PA-induced SLE may ultimately be related to the functional loss, by either a genetic or pharmocologic mechanism, of a small population of suppressor T cells which have been linked to autoimmunity. (Tannen RH, Cunningham-Rundles S, Good RA: Unpublished data.)

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DISCUSSION

Dr. Talal: It may be possible to augment the difference between C57BL and A/J by the introduction of a new element, namely, the drug procainamide. Your experimental manipulation, in this case by giving the drug, in another case the breeding experiment, may be intensifying a defect which perhaps is only minimal in its natural setting. In rheumatology we think in terms of multifactorial etiologies of disease. This would be an exteremely important point.

Dr. Weber: This apparent augmentation may be due to the way the data are presented. In Figures 4 and 5, which showed the increase in the titer with time for slow and rapid versus the two parental strains, the titer of the animal is noted at a given point in time, for example at the end of 35 or 36 weeks. This method of determination is the way that Ten Veen and Feltkamp expressed their information. We found, how-

ever, that by measuring titers throughout the course of the lifetime of the animals when they were taking procainamide, we could get more information from the data. We had 8 data points instead of one for analysis. The actual division point in time when the animal began to respond changed a bit; that is, in the case of the slow or the A/J parental lines, it may have taken a few weeks before any response occurred. We could then conclude that there was no difference in that part of the response from no exposure and include that in either the weak responder or non/non-responder stage. Then it was clear from the time course that the response began to increase quite rapidly in the slow animals.

Dr. Alarcon-Segovia: There is a study that may be pertinent in which A/J mice were changed from one environment to another and started producing antinuclear antibodies.

Dr. Shulman: In the early sixties Friou obtained pooled sera from 9 different inbred strains at the Jackson Laboratory and reported that the A/J strain was the only 1 of these 9 strains that had antinuclear antibody, which was found in about 25% of the animals (Friou GJ, Teague PO: Arthritis Rheum 6:773–774, 1963). I then requested some A/J animals from the Jackson Laboratory. As a genetically related strain the A Heston was chosen, which you showed as your second strain. We also asked for a genetically unrelated strain, the C57BL/6J.

We found a 25% incidence of ANA in the A/J, 22% in the C57BL/6J, and 39% in the A/He, the genetically related strain. So we suspected an environmental factor. We found that the incidence of ANA was much lower in mice from Cumberland Farms in Tennessee, 5% and 3% compared to the 22% and 39% in the animals from Jackson Labs (Shulman LE et al: Arthritis Rheum 7:78, 1964).

Dr. Heston at the Cancer Institute informed us that he had donated these strains to the Cumberland Farms and thus considered that the genetic makeup was the same. We cross-grafted skin from the A/He strain animals from the Cumberland Farms and the Jackson Labs, and grafts were successful. That experience may indicate some environmental differences from producer to producer. Perhaps, Dr. Weber, some of your differences with Dr. Feltkamp might be related to animal source. Are your animals from Jackson Labs?

Dr. Weber: Yes, they were. Dr. Feltkamp's animals were from Jackson Laboratories and were shipped to Holland and maintained there, so the environment was not the same. Certainly the enzymes responsible for drug metabolism are highly affected by the environment. In this case, I do not know of any available definitive evidence.

Dr. Talal: Dr. Lloyd Old at the NIH Cancer Institute and I were interested in the age and sex dependent development of antiDNA and antiRNA antibodies. We had our NZB and B/W mice in two buildings: our main colony in the Clinical Center and a second colony in Lloyd Old's building at the Cancer Institute. We took blood samples from both groups and analyzed them separately.

There was a significantly earlier onset of development of autoantibodies and a higher incidence of autoantibodies in the mice housed in the Cancer Institute building as compared with those in the Clinical Center. The other work in the Cancer Institute building dealt extensively with oncogenic viruses.

This is an anecdote, but one factor we considered important for this difference was the presence of oncogenic and other viral agents in the Cancer Institute Building.

Dr. Weber: All of the mice in my studies were maintained in a separate room throughout the experiment. So the environment was fairly constant across the group. The differences seen here should be related to drug exposure.

Dr. Tan: Dr. Weber, there appears to be a difference in hepatocyte activity between mouse and rabbit.

Dr. Weber: There is a big difference in acetylating capacity between mouse and rabbit. There can be a several hundredfold difference in liver activity between a rapid and slow acetylator rabbit. The difference between A/Js and C57s (the absolute level of activity is lower in the mouse strains) for example, with amino fluorine as a model substrate is about tenfold. Also, it is clear that substrate specificity is involved because there is no in vitro difference with procainamide in these mouse strains.

Dr. Dubroff: A critical factor is whether incubation was in darkness or light. It should have been in absolute darkness; otherwise tremendous variability results since this tissue culture reaction is photochemical.

Dr. Weber: We were concerned about the capability of the hepatocytes to repair in the rapid and slow acetylator rabbits. So we ran an ultraviolet control. The capacity of the hepatocyte to repair DNA was precisely the same in the rapid and the slow acetylators. This large difference does exist for the two substances, but the differences are in opposite directions.

Dr. Dubroff: A photochemical reaction is markedly enhanced by certain wavelengths of light. In this case, it is 290 nanometers to about 330 nanometers. If you did not prepare these in absolute darkness, your reaction would be in part photochemical. Another problem is the incubation media. Hydralazine is susceptible to degradation in the presence of divalent cations.

Dr. Weber: We did put hydralazine in and the difference is related to hydralazine dose, although we don't know specifically what is occurring or how many different steps in the breakdown of hydralazine may have taken place before it produced damage. These experiments were performed in the absence of light.