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Evaluation of a Lymph Node Proliferation Assay for its Ability to Detect Pharmaceuticals with Potential to Cause Immune-Mediated Drug Reactions

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Abbreviations: LLNA, Local Lymph Node Assay; LNPA, Lymph Node Proliferation Assay; pLPNA, popliteal Lymph Node Proliferation Assay; PLNA-RA, Popliteal Lymph Node Assay Reporter Antigen; S.I., Stimulation Index; PB, Phenobarbital; MET, Metformin; STZ, streptozotocin; SMX, sulfamethoxazole; OFX, ofloxacin; CLN, clonidine; CPZ, chlorpromazine; PA, procainamide; NEV, Nevirapine; ZOM, zomepirac; LAM, lamotrigine; ABA, abacavir.

Hypersensitivity reactions to systemically administered drugs cannot be predicted using available preclinical models. This research is a collaborative project to evaluate the ability of the Lymph Node Proliferation Assay (LNPA) to predict systemic hypersensitivity caused by pharmaceuticals. The assay design is a modification of the Local Lymph Node Assay with the major modification being injection of the test substance subcutaneously to achieve a known systemic exposure to the drug. Fourteen compounds were evaluated in the LNPA. These were two clinically negative drugs (Metformin, phenobarbital), an assay positive control (streptozotocin), eight human hypersensitivity positive drugs (sulfamethoxazole, procainamide, clonidine, ofloxacin, nevirapine, abacavir,

lamotrigine, zomepirac), and 3 investigational drugs (CM40874, CM40954 and CM40420), one of which caused hypersensitivity in primates. Hypersensitivity-positive drugs were classified as such based on at least two of three independent data sources: U.S. FDA postmarketing database, drug labeling information, and clinical trial data. All drugs were tested in multiple laboratories for a total of 2–12 evaluations per compound. The pure drug substance was used for testing if it could be obtained commercially, otherwise the marketed drug formulation was used. Neither of the negative control drugs showed a positive reaction in the test system. Four of the eight hypersensitivity positive drugs showed a mixed or positive reaction. Two of the three investigational compounds gave a positive response. A smaller number of LNPAs were run concurrently using footpad injection and evaluation of the popliteal lymph node and gave generally comparable results. Additional development may increase the reproducibility of the assay and facilitate detection of drugs that require metabolic activation to become allergenic, or drugs for which there is dose-limiting toxicity. The data suggest that this method might be useful as a first-line screen to identify candidate drugs that are more likely to cause a high prevalence of human drug hypersensitivity.

INTRODUCTION

The process of developing a new chemical entity (NCE) into a marketed pharmaceutical has many routes to failure and is very costly. Overall, approximately 80% of NCEs that enter clinical trials do not become approved drugs, and 20% of the failing drugs (15% of all NCEs) do so because of safety issues (DiMasi, 2001). Many of these safety failures in clinical trials can be viewed as a failure of preclinical testing methods. A major portion of the preclinical testing in animals is designed to identify potential human toxicities, and their safety margin during initial human exposures. Animals are generally good predictors of human responses, however for some types of toxicity, animal models are not currently adequate to predict potential human toxicity. Drug hypersensitivity was identified in an ILSI-HESI sponsored study as the area where animal models are least able to predict human responses (Olson et al., 2000). The methods available for testing NCEs for their potential to cause drug allergy are very limited. The traditional methods for detecting sensitization potential utilize various guinea pig skin sensitization methods. These have been shown to perform well for predicting skin sensitization caused by industrial or environmental chemicals (Klecak et al., 1977; Klecak, 1985; Maurer, 1996), and while they have been used for evaluation of pharmaceuticals since the 1980s, there is little data to support their ability to predict systemic drug hypersensitivity. In fact, an analysis comparing the results of guinea pig sensitization assays with human drug adverse event reports suggestive of drug hypersensitivity shows no statistical association between the two sets of data for systemically administered drugs (Weaver et al., 2003). Other published methods such as the popliteal lymph node assay (PLNA) (Kammuller et al., 1989) and the reporter antigen version (RA-PLNA) (Albers et al., 1997) may have potential, but there is not enough published data on the responses of these methods with drugs to determine their applicability as techniques for hazard

identification. There are also animal welfare considerations (see below) that preclude the use of this method for routine screening.

More recently the mouse local lymph node assay (LLNA) was extensively evaluated by ICCVAM (The Interagency Coordinating Committee on the Validation of Alternative Methods) and accepted as an alternative to the guinea pig assays for prediction of skin sensitization (Dean et al., 2001; Basketter et al., 2002). However, this method is not directly suitable for evaluation of systemically administered drugs since the LLNA method utilizes topical exposure to the test substance which results in unknown and variable levels of local and systemic exposure. In this study, the LLNA method was modified to administer drug by subcutaneous injection of test compounds. The subcutaneous injection method results in known systemic exposure and also avoids the complex issues of oral bioavailability, hepatic metabolism, and oral tolerance. Furthermore, this method of drug delivery was also chosen as a route of administration that appears to be more humane than the footpad injection used in the popliteal lymph node assay. The animal welfare concerns associated with inflamed footpads as observed with the injection of certain drugs, for example zomepirac and glafenine (Gutting et al., 2002), are avoided with the head area injection location. As discussed below, inflammation was seen with some compounds at the injection site. The modified LLNA method, referred to in this paper as the Lymph Node Proliferation Assay (LNPA), was evaluated in a collaborative project to determine if this method would be able to predict the ability of drugs to induce drug allergy in humans after systemic exposure. We evaluated 2 known negative compounds, an assay positive control, 8 known positive drugs and three investigational compounds in the LNPA assay. We found that the assay gave positive responses for 4 of 8 compounds known to be positive in humans, 2 of 3 investigational compounds, and did not respond to the 2 negative drugs.

MATERIALS AND METHODS

Selection Criteria for Drugs

The drugs selected for inclusion in this study had to meet several criteria. Drugs were selected in three categories, hypersensitivity negative, assay positive control, and hypersensitivity positive. The hypersensitivity positive drugs were selected because they were shown to cause human drug allergy based on at least 2 independent sources of information. These sources were: U.S. FDA postmarketing data, drug labeling information, and clinical trial data. Clinical negative drugs were found to have very low reporting index data for all types of drug allergy adverse event reports in U.S. FDA postmarketing data. The data in this compilation are for all dosage forms and routes of administration. A summary of the adverse event reporting index data for the marketed drugs used in this study is shown in Table 1. We included sulfamethoxazole and procainamide to determine if the need for metabolism observed with the PLNA would also occur with the LNPA. Three investigational drugs (cephalosporin antibiotic class) were selected since the parent of the class (CM40874)

TABLE 1
Association among IDHR adverse events for test pharmaceuticals

Generic name	Adverse event reporting index ¹ for									
	Rash	Pruritis	Urticaria	Application site reaction	Leukopenia	Edema face	Allergic reaction	Asthma	Injection site reaction	Anaphylactoid reaction
Hypersensitivity positive										
Abacavir	<u>43²</u>	<u>66</u>	<u>24</u>	0	<u>8.6</u>	0.8	<u>124</u>	2	0.8	<u>4</u>
Clonidine	<u>1065</u>	<u>777</u>	<u>116</u>	<u>1441</u>	<u>9.5</u>	<u>13</u>	<u>48</u>	<u>15.3</u>	0	<u>4</u>
Lamotrigine	<u>79</u>	<u>18</u>	<u>5</u>	0	<u>14.6</u>	<u>13</u>	<u>9</u>	1	0	0.5
Nevirapine	<u>508</u>	<u>179</u>	<u>34</u>	0	<u>34</u>	<u>41</u>	7	7	0	7
Ofloxacin	<u>54</u>	<u>27</u>	<u>27</u>	0.2	<u>7</u>	<u>23</u>	<u>15</u>	<u>8</u>	<u>2.6</u>	<u>22</u>
Procainamide	<u>246</u>	<u>108</u>	<u>60</u>	0	<u>252</u>	<u>42</u>	<u>42</u>	0.2	<u>12</u>	<u>0</u>
Sulfamethoxazole	<u>44</u>	<u>15</u>	<u>29</u>	0	0	<u>15</u>	<u>29</u>	0	0	<u>15</u>
Zomepirac	<u>175</u>	<u>178</u>	<u>149</u>	0	1.6	<u>56</u>	<u>71</u>	<u>52</u>	0	<u>107</u>
Count of + Drugs	8	8	8	1	6	7	8	4	1	5
NC ²										
Metformin (nc)	0	0	0	0	0	0	0	0	0	0
Phenobarbital (nc)	0.9	0.9	0.6	0.3	0.6	0.4	0	0.1	0	0.2
APC ³										
Streptozotocin (apc)	0	0	0	0	<u>11.8</u>	0	0	0	<u>36</u>	0

¹Reporting index of adverse event reports per million cases of drug shipped.

²An RI of >3 is classified as positive and is in bold text, strongly positive values (RI > 20) are also underlined.

³(NC)—negative control.

(APC)—assay positive control.

produced hemolytic anemia, edema, glomerular lesions, and other manifestations of delayed-onset systemic hypersensitivity symptoms in primate studies, causing the class to be dropped. An immunological mechanism was confirmed by immunofluorescence that revealed extensive immune complexes in the basal medulla of the renal glomeruli (Dean, personal communication). The investigational compounds CM40420 and CM40954 are closely related members of the same chemical family. The assay positive control, streptozotocin, has been shown to induce strong positive responses in mice (Krzystyniak et al., 1992; Choquet-Kastylevsky et al., 2000; Nierkens et al., 2004).

Drugs

The drugs streptozotocin, procainamide, clonidine, ofloxacin, zomepirac, sulfamethoxazole, phenobarbital, and metformin were obtained from Sigma Chemical Co. (St. Louis, MO). These drugs were made up as a solution or suspension in phosphate-buffered saline. For nevirapine, abacavir, and lamotrigine, the marketed pharmaceutical preparations were used. Nevirapine (Boehringer-Ingelheim, Ridgefield, CT) was injected as the oral suspension with a concentration of 10 mg/ml. Abacavir sulfate (GlaxoSmithKline, Research Triangle Park, NC) was injected as an oral solution at 20 mg/ml. Lamotrigine (GlaxoSmithKline, Research Triangle Park NC) was obtained as 5 mg dispersible tablets. The tablets were dispersed in PBS and then sonicated for 30 minutes at room temperature in a bath sonicator. The investigational compounds CM40874, CM40954 and CM40420 were a generous gift of Sanofi-Aventis Inc. (Malvern, PA). These drugs were also injected in solution in phosphate-buffered saline (PBS). All drugs were purchased directly by each participating laboratory.

Mice

Female mice of 5–7 weeks of age were obtained by the various laboratories participating in this study from these suppliers: BALB/c were obtained from: Harlan Sprague-Dawley (Indianapolis, IN; or Horst, The Netherlands), Jackson Laboratory (Bar Harbor, ME), Charles River Laboratories Europe, (l'Arbresle France), Charles River Laboratories, (Wilmington, MA; or Raleigh, NC), or Taconic, (Germantown, NY). CBA/N mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN), and CBA/Ca mice were obtained from Jackson Laboratory, (Bar Harbor, ME). C57Bl/6 mice were obtained from Charles River Laboratories (Raleigh, NC). Mice were 6–10 weeks old at the time of the actual experiments.

Dose Ranging Studies

These studies were performed in a single laboratory and the results were used to select the doses used by all participating laboratories. Starting doses for the dose ranging studies were estimated from published data and information in drug labels. The doses were escalated in doubling steps (e.g., 1, 2, 4 mg/kg/day). Mice were dosed for 3 days with daily observation and weighing. Maximum doses were selected that did not cause weight loss

or serious clinical symptoms. It should be noted that occasional toxicity was noted at the high dose in the main study and is mentioned in the results section for the specific drugs where this was observed. For drugs where the pharmaceutical preparation was used, toxicity was never limiting and drugs were administered at the maximum practical volume of drug solution or suspension (100 μ l).

Animal Study Protocols

All experiments were performed in compliance with the animal study regulations at each individual organization, and with the approval of the animal care and use committee at each investigators' respective organization.

LNPA Protocol

The LNPA method was derived from the ICCVAM approved LLNA protocol (<http://iccvam.niehs.nih.gov/methods/llnadocs/LLNAProt.pdf>.) The drug was made up as a solution or suspension at a concentration that allowed it to be administered subcutaneously in a volume of 50–100 μ l. The drugs were administered using a 0.5 ml syringe with a 25-gauge (or smaller) needle. For the cervical lymph node version of the assay, the point of injection was on the top of the head, in the midline, between the eyes. The needle was pointed toward the back of the animal so that the end of the needle was just forward of the point between the ears. The drug solution or suspension was slowly injected so that the bulk of the liquid was deposited between or just forward of the ears. The location of injection is important because if it is too far to the rear, the response in the draining superficial cervical lymph node is reduced. Three concentrations of drugs were used, the maximal tolerated (MTD) or maximum practical dose, half of MTD, and quarter of MTD. PBS was used as the vehicle control for all drugs since the specific composition of nondrug components of the pharmaceutical preparation is not publicly available.

Groups of 5 mice per dose were injected daily for 3 days (Wed–Fri) and then rested for 2 days. On day 6 of the experiment (Mon), the mice were injected IV in the tail vein with 200 or 250 μ l of PBS containing 20 microCi of ^{3} H-methyl-thymidine. After 6 hours, the mice were sacrificed and both superficial cervical lymph nodes were removed. Cells were prepared and radioactivity was measured as described in the LLNA method.

The studies were performed by multiple labs using this specific protocol. An experiment was defined as an evaluation with 1 drug at 3 dose levels. All approved drugs were evaluated by 3–7 different laboratories and may have been tested more than once at a single laboratory. The investigational compounds were tested once each at 2 or 3 laboratories due to the limited amount of compound available for testing.

Popliteal LNPA Protocol

Studies with the popliteal lymph node were performed by injecting the test material into one hind footpad in a volume of 50 μ l (Albers et al., 1997). The method was otherwise identical

to the LNPA except that the appropriate draining popliteal lymph node was collected for radioactivity measurement.

Data Analysis

The data are reported as DPM/mouse and group mean DPM is calculated for each dose group. The stimulation index (S.I.) is calculated from the dose group mean DPM divided by the mean DPM for the vehicle control concurrent with that experiment. A single run of each dose group of 5 mice in one laboratory was considered one experiment. Based on the extensive statistical analysis conducted as a part of the ICCVAM peer review of the LLNA, a stimulation index of 3.0 was found to be the optimal cutoff for a significant response. See the discussion section for consideration of this value and comparison with mean DPM + 3 SD as a positive response criteria. A positive call was arbitrarily set as needing both an S.I. of 3.0 or higher and a dose-response trend.

RESULTS

The summary of the results for this study is shown in Table 2. The negative and assay positive control compounds behaved much as expected. Among the 8 compounds for which clinical drug hypersensitivity was observed in postmarketing adverse event data, the results can be placed into 3 categories, generally positive, mixed results, and generally negative responses.

Assay Positive Control

Streptozotocin was evaluated as a possible assay positive control compound. The results demonstrate strong positive responses in 5 of 7 laboratories. The reasons for the inability of 2 laboratories to observe positive responses for this compound are not known.

Clinical Negative Control Compounds

We used metformin and phenobarbital as negative compounds because the human clinical data showed little, if any evidence of drug allergy. Metformin produced no positive responses in any laboratory. The results for phenobarbital are also largely negative, however, one laboratory reported a positive S.I. of 3.27 at the high dose only, and a dose-response trend. This finding was not observed in the other laboratories and is considered an outlier.

Clinical Positive, LNPA Positive Compounds

Two compounds showed generally positive responses in the LNPA. The data for the drugs nevirapine and lamotrigine showed strongly positive responses from most or all of the laboratories testing these compounds (Table 2).

Clinical Positive, LNPA Mixed Response Compounds

Two of the 8 compounds tested (ofloxacin and abacavir), fell into this category. The responses observed at the high dose of abacavir should be viewed with caution as some, but not all,

laboratories observed major inflammation at the injection site. Because the clinical preparation of the drug is only available at a fixed concentration, the high dose was given as 2 injections of 100 μ l with 6–8 hours between injections. For both ofloxacin and abacavir, the results were positive in some experiments and negative in other experiments. This result was seen both between laboratories and among different experiments within the same laboratory.

Clinical Positive, LNPA Negative Compounds

Among the 8 compounds known to have significant human clinical effects based on adverse event reporting, 4 showed generally negative responses in the LNPA as performed in this study (sulfamethoxazole, procainamide, zomepirac, and clonidine). Among this group of 4, only clonidine gave any suggestion of a positive response, and then only at the high dose. Higher doses of clonidine could not be investigated due to toxicity resulting from excessive pharmacological activity of this drug.

Investigational Compounds

The parent compound in this class (CM40874) was found to cause hemolytic anemia, Coomb's positivity and delayed-onset glomerular nephritis, all manifestations of immune-mediated hypersensitivity reactions in a primate study. This drug was found by 2 labs to have SI values over 3.0 in the LNPA. Due to a very limited amount of material available, a dose response could not be established. Two structurally-related compounds that had not been evaluated in animals were also tested; one (CM40954) was clearly positive while the other molecule (CM40420) was negative. These data would suggest that this assay might offer a potential method of screening new investigational compounds for their potential to produce systemic immune hypersensitivity.

Strain Comparison

Most laboratories used BALB/c mice, however, 2 laboratories used CBA strains of mice. There are no apparent differences in the responses of the CBA mice compared to the BALB/c mice. The average difference in S.I. across all drugs was 1.19 with a standard deviation of 5.96. There were a limited number of experiments using C57Bl/6 mice. These also show no particular pattern of responsiveness that is different from the BALB/c mice, although the C57Bl/6 mice appeared to be less sensitive in terms of dose response. The average difference here was –1.28 with a standard deviation of 4.52.

Popliteal LNPA Data

The results for a limited set of experiments using the popliteal lymph node variant of the LNPA are shown in Table 3. Results are in agreement with the LNPA except for those obtained using phenobarbital.

DISCUSSION

The results of this study suggest that the LNPA, in its current form, has significant promise for detection of the potential

TABLE 2
Summary results with lymph node proliferation assay

Drug name	Dose (mg/kg/day)	Stimulation index ¹ (Average $\pm$ S.E.M.)	Positive calls ² by S.I./total # experiments
Streptozotocin	25	6.70 $\pm$ 2.27	5/7
(Assay positive control)	50	9.61 $\pm$ 2.83	
	100	24.16 $\pm$ 12.03	
Metformin	25	1.08 $\pm$ 0.27	0/9
(Negative)	50	1.18 $\pm$ 0.20	
	100	1.30 $\pm$ 0.18	
Phenobarbital	25	0.96 $\pm$ 0.12	1/6
(Negative)	50	1.02 $\pm$ 0.16	
	100	1.27 $\pm$ 0.40	
Nevirapine	16	2.65 $\pm$ 0.45	4/5
	33	4.62 $\pm$ 0.91	
	66	6.87 $\pm$ 2.13	
Lamotrigine	15	6.04 $\pm$ 1.39	4/4
	30	7.64 $\pm$ 1.81	
	60	15.27 $\pm$ 6.25	
Abacavir	66	2.18 $\pm$ 0.59	4/5
	133	2.90 $\pm$ 0.78	
	266	5.03 $\pm$ 0.79	
Ofloxacin	25	1.45 $\pm$ 0.18	6/12
	50	3.46 $\pm$ 0.87	
	100	4.40 $\pm$ 1.19	
	200	9.37 $\pm$ 3.52	
Clonidine	5	1.32 $\pm$ 0.30	2/6
(dose limiting toxicity)	10	2.03 $\pm$ 0.32	
	15	2.40 $\pm$ 0.53	
Zomepirac	6.25	0.91 $\pm$ 0.11	0/4
(dose limiting toxicity)	12.5	1.00 $\pm$ 0.09	
	25	0.95 $\pm$ 0.20	
Sulfamethoxazole	25	1.11 $\pm$ 0.05	0/6
(metabolism needed)	50	1.15 $\pm$ 0.11	
	100	1.05 $\pm$ 0.12	
Procainamide	10	1.33 $\pm$ 0.30	0/3
(metabolism needed)	25	1.17 $\pm$ 0.32	
	50	1.15 $\pm$ 0.16	
CM40874	12.5	nd, 3.2	2/2 ³
	25	6.15, 3.1	
	50	6.02, 3.5	
	100	6.84 , nd	
CM40954	25	2.45 $\pm$ 1.24	3/3
	50	5.05 $\pm$ 1.08	
	100	7.24 $\pm$ 1.43	
CM40420	25	1.92 $\pm$ 0.55	0/3
	50	2.14 $\pm$ 0.29	
	100	2.05 $\pm$ 0.31	

¹The stimulation index is pooled across all laboratories and all experiments within laboratories.

²Positive call requires both an S.I. of 3.0 or higher and a dose response trend across all dose groups and is also pooled across all laboratories and all experiments within laboratories.

³Dose response could not be evaluated due to limited availability of compound.

TABLE 3

LNPA popliteal variant—Individual experiment data for injection of drug into the hind footpad and collection of the popliteal lymph node

Drug	Lab #	Stimulation index		
		Low ¹	Medium	High
Phenobarital (negative)	1	1	1	4 ²
	2	nd ³	1.3	0.3
Metformin (negative)	1	1	1	2
	2	0.8	0.9	1.0
Streptozotocin (assay positive control)	1	17	39	63
	2	5.9	12.1	30.8
Ofloxacin	1	1	2	7
	2	1.7	1.6	1.4
Clonidine	1	nd	nd	nd
	2	0.5	0.5	0.3
Sulfamethoxazole	1	2	3	2
	2	nd	nd	nd
Procainamide	1	nd	nd	nd
	2	0.9	1.4	1.1

¹Dose of drug is the same as shown in Table 2.

²Bold text indicates S.I. value above the significance cutoff value of 3.0.

³nd—not done.

to induce systemic hypersensitivity. However, it is clearly not able to reliably identify all drugs that may cause significant human drug hypersensitivity based on comparison with post-marketing or clinical trials data. Additional refinements may improve the predictive value of this method. In its current form, it may be adequate as a first-line screen to assist in identifying drugs with allergenic potential among a series of new drug candidates.

The selection of both negative and positive marketed test compounds to evaluate this method was a complex process balancing many selection criteria. These included: known clinical experience from the FDA adverse event database, clinical trial data reported in drug labeling, warnings in drug labeling, and reports in the scientific literature. Additional considerations included the commercial availability of the compound as a pure drug substance, the availability of data to set starting doses, the extent of reported use of the compound, and the aqueous solubility of the drug. The clinically-negative drugs phenobarbital and metformin were selected based on high usage and extremely low reported numbers of immune-related adverse events. Adverse event data were normalized to a reporting index (R.I.), which is the number of adverse event reports per million cases of drug shipped. For both compounds, the R.I. for the selected immune-related adverse events was less than 1, which is very low for drugs with such high usage. Both drugs had shipping values above 2.4 million cases/year for the time period examined.

The hypersensitivity-positive drugs were included for a variety of reasons. Zomepirac was withdrawn from the market in the U.S. due to drug hypersensitivity in humans, has high RI values in adverse event data (Table 1), and numerous publications on clinical hypersensitivity reactions (Strom et al., 1987; Corre and Spielberg, 1988; Spiers, 1988). Nevirapine, lamotrigine, and abacavir have black box warnings for potential drug hypersensitivity in the drug label as well as high R.I.s in postmarketing data (Table 1). Ofloxacin and sulfamethoxazole have specific warnings in the drug label for drug hypersensitivity and strong R.I. data (Table 1). In addition, they are members of the fluoroquinolone and sulfanilamide families, both of which are known to have significant hypersensitivity issues. Clonidine was selected due to its high R.I. values in many of the immune-related adverse event categories. Procainamide was included to determine if the negative findings reported with the basic PLNA assay (Patriarca et al., 1993) would also be found with the LNPA. In addition, this drug has especially high R.I. values (>500) in autoimmune adverse event categories such as anti-nuclear antibodies and lupus erythematosus syndrome. We were also interested to learn whether the more acute systemic exposure via subcutaneous injection would result in sufficient metabolism of sulfamethoxazole and procainamide, both of which are thought to require metabolic activation to be allergenic. All of the positive drugs selected had significant R.I. values in the adverse event terms rash, pruritis, urticaria, and edema-face (Table 1). Additional terms where a majority of the drugs have significant R.I. values are anaphylactoid reaction (includes anaphylaxis), angioedema, and Stevens-Johnson syndrome (Table 1).

The drug streptozotocin was selected as an assay positive control due to the strong positive reactions reported in the PLNA with this compound (Krzystyniak et al., 1992; Choquet-Kastylevsky et al., 2000; Nierkens et al., 2004). It was expected that streptozotocin would be strongly positive in the LNPA. This drug has been shown to stimulate interferon-gamma and IL-4 from T_H2 lymphocytes (Nierkens et al., 2004) and also to directly stimulate macrophages (Nierkens S, thesis). The drug is used solely for the treatment of pancreatic cancer in humans, where it is not reported to cause significant drug hypersensitivity. The minimal number of immune-associated adverse event reports in the FDA database and in published data (Litt, 2002) may be due to the small numbers of patients exposed to the drug. In addition, since cancer patients are often treated with multiple drug regimens, assignment of an adverse event to a specific drug is difficult. The underlying disease and the other chemotherapy drugs may also alter immune responsiveness to the drug. The reason for the failure of this compound in 2 labs is not known but could possibly be related to variations in injection site. As these labs were unable to continue participating in the project, this and other possible explanations were not explored.

The clinically-negative drugs metformin and phenobarbital drugs were also negative in the LNPA with a single exception each. One of the 5 laboratories reported a value of >3.0 for

metformin at the low dose, but not at the mid or high doses. This observation was not repeated in this or other laboratories, and therefore, we consider this an outlier and not a biologically significant observation. The data for phenobarbital showed 1 of 6 values over 3.0 for the high dose only. As only one other lab had an SI over 1.0 for the high dose, we consider this to be an outlier. We conclude that these compounds are clearly not able to provoke a statistically valid positive response overall. It is possible that the assay variability in general might be higher with the LNPA than the LLNA, which might require adjustment of the threshold value. This can only be resolved with a significant amount of additional data, preferably directly comparing performance of the 2 assays.

Among the drugs tested that are known to cause significant drug hypersensitivity in humans, three patterns of response were observed, a mostly positive response, a mixed response, and a mostly negative response.

The first set of marketed drugs, nevirapine and lamotrigine provided responses that were most consistently positive. These drugs are among those known to cause prevalent and often severe drug allergy symptoms. It appears that the assay may be most reliable with this strongly positive category of compounds.

The next group of drugs (abacavir and ofloxacin), which showed a mixed positive response, were the most problematic to explain. Inflammatory responses consisting of redness, swelling, and hair loss at the injection site were observed by some laboratories with the high dose of abacavir. The positive responses at this dose might in part be related to this irritation and/or inflammation. During the LLNA validation studies the data showed that the irritant sodium dodecyl sulfate produced a false positive response in that assay due to inflammation (Loveless et al., 1996). Inflammatory changes however, were not seen with ofloxacin. The unpredictable responses seen with these two drugs, which were also observed between replicate runs in the same laboratory make it difficult to place high reliance on the results of an individual run for these two agents. Despite careful evaluation of the individual studies, no reason for the variability in the response could be identified. Variability in the responses was not associated with performance with the assay positive control streptozotocin, nor was it associated with particularly high or low values for the negative control.

The human positive drugs that provoked no response in the LNPA were sulfamethoxazole, procainamide, zomepirac, and clonidine. The dose-limiting toxicities seen with clonidine and zomepirac may have prevented administration of doses high enough to produce a positive response. Clonidine did show a dose response and 2 labs did have an SI over 3.0 at the high dose. Alternately, there may be a requirement for metabolic activation for these drugs, or some other factor such as a requirement for specific histocompatibility antigen haplotype. After the experiments with sulfamethoxazole were completed, it was reported that in the classical PLNA the negative response could be converted to a significant positive response after metabolic activation with S9 (Choquet-Kastylevsky et al., 2001). This

explanation may also apply to procainamide where S9 treatment also produced a positive PLNA response (Katsutani and Shionoya, 1992; Patriarca et al., 1993). A study using alternate metabolites of procainamide showed that the hydroxylamine metabolite was active in a PLNA assay but that the N-acetyl metabolite and the parent drug were inactive (Kubicka-Muranyi et al., 1993). The failure to observe a response to sulfamethoxazole or procainamide implies that we failed to get sufficient metabolic activation of these drugs in our test system. It may be that others of the negative or mixed response group in this study would show a positive response if metabolites were tested in addition to the parent drug, in some cases, specific metabolites can be tested as was done for procainamide (Kubicka-Muranyi et al., 1993). For the current generation of investigational drugs, metabolites are often synthesized by the sponsor and could be tested. However, for older marketed drugs, the metabolites may not be as well characterized and are less likely to be available for testing. There is a clear need for developing a simple method to generate metabolites in sufficient quantity to be used with *in vivo* test methods such as the LNPA.

We were able to obtain 3 investigational new drug compounds from a cephalosporin antibiotic family of drugs that were terminated from development due to adverse hypersensitivity effects seen in primate and rat studies following administration of the lead compound, CM40874. This compound gave positive S.I. values in the LNPA assay. We were not able to determine the dose response due to very limited availability of this compound. One of the 2 related compounds, CM40954, was found to be clearly positive in the LNPA and the other was negative. These compounds gave varying levels of structural alerts in the Derek software program but could not be evaluated by Topkat due to lack of structural coverage. These results suggest that the assay may have some intrinsic value in screening new investigational compounds. Inclusion of compounds that have failed during development due to adverse immune effects adds significant value to the evaluation of the performance of the LNPA.

The issue of poor drug solubility does not appear to have clear influence on the test results, as abacavir was administered as a solution and ofloxacin as a suspension and both gave mixed responses. In the highly-active group, nevirapine was administered as a very finely divided pharmaceutical suspension. Also, lamotrigine was injected as a viscous suspension of dispersable tablets. Drug solubility is not required for activation as suspensions were active. It can be argued that suspensions might be more immunogenic due to the activation of macrophages by the particles of the suspension. The activity of abacavir argues that this is not required. However for some drugs, insolubility may have prevented adequate dosing. This question of the influence of drug (in)solubility cannot be answered with the data available in this study.

At this point, there is insufficient experience with the LNPA to identify a specific value as representing the best cutoff S.I. value to distinguish a true positive from a true negative response. Therefore we have adopted the standard LLNA threshold of

greater than or equal to 3.0 for our initial evaluation of the LNPA. The value of 3.0 was retrospectively found to be a statistically appropriate value for the LLNA based on extensive data analyzed during the validation review process (Dean et al., 2001; Basketter et al., 2002). Examination of the data from individual experiments shows that a dose response was observed for nearly all drugs where an S.I. value above 3.0 was seen. This appears to support the utility of this cutoff value of 3.0 for the LNPA. In addition, for a subset of the data (18 drug runs $\times$ 3 doses/run), we compared the use of the S.I. cutoff of 3.0 with a threshold of the mean DPM + 3 standard deviations as a measure of positive response. There was complete agreement between the S.I. of 3.0 and mean DPM + 3 SD as indicating a positive response at all individual dose levels (data not shown).

A secondary aim of this study was to evaluate the responses of several strains of mice to determine if there were any strain-specific effects. The majority of the data was collected using BALB/c (MHC d haplotype) mice, with some studies being performed with CBA (MHC k haplotype) or C57Bl/6 mice (MHC b haplotype). The data indicate that neither strain has a response pattern that was different from that seen with the BALB/c mice. In addition, mixed responses were seen with both BALB/c and CBA mice.

Two investigators evaluated the effect of injecting the drug into the hind footpad of the mouse for comparison to the head site of injection. The results, shown in Table 3, are generally consistent with the results seen with the head injection site. These results suggest that either route of administration may be used. However, other considerations such as animal welfare issues, may influence the choice of the head vs. the footpad as the route of administration.

In parallel with these studies, some of the same drugs were analyzed using the Reporter Antigen (RA)-PLNA method in one of our laboratories and compared with ear injection with RA (Albers et al., 1997). These studies are reported in detail elsewhere (Nierkens et al., 2004). In summary, the RA-PLNA and the ear injection assay, designated RA-auricular lymph node assay (RA-ALNA) showed significant responses against streptozotocin and ofloxacin, but not metformin, sulfamethoxazole, phenobarbital, or procainamide. These results are similar to those seen with the LNPA.

Our data suggest that the LNPA is a promising method that might benefit from additional development to improve its predictive value, especially with drugs that may require metabolic activation. It is possible that more than one type of metabolic activation system will be needed. An additional area for research is the development of alternate endpoints, especially non-radioactive methods. Alternate endpoints should be closely related to the stimulation of the immune system, for example, antibody responses, alterations in lymphocyte activation markers, or cytokine production. In addition, a significantly larger number of positive and negative compounds will need to be evaluated to get a more accurate estimation of the true predictive value and utility of this assay. The data presented here suggest that the

TABLE 4
Comparison among human adverse event data and lymph node assays

Drug	LNPA ¹	popLNPA	Human post marketing
Metformin	— ²	—	—
Phenobarbital	—	+/-	—
Streptozotocin	+	+	—
Zomepirac	—	n.d.	+
Sulfamethoxazole	—	—	+
Procainamide	—	—	+
Clonidine	—	—	+
Ofloxacin	+/-	+/-	+
Abacavir	+/-	n.d.	+
Nevirapine	+	n.d.	+
Lamotrigine	+	n.d.	+

¹LNPA—data from this study with cervical LNPA; popLNPA—data from this study with popliteal LNPA; Human Postmarketing—Hypersensitivity adverse event data from FDA postmarketing data.

²— negative results, +/- mixed results, + positive results, n.d.—not done.

LNPA method might provide a useful screen to identify new drug candidates with allergenic potential.

The summary results with the standard and popliteal LN variant of the LNPA are compared with the available results for human clinical experience, as represented in the FDA adverse event data (Table 4). When compared against the adverse event data, none of the current assays are able to predict hypersensitivity reactions with a high level of accuracy. The reasons for this are not known at this time. There are several factors that may be reducing the predictive power of the method. It is known that there are multiple routes to initiate hypersensitivity reactions (Lerch and Pichler, 2004). It is entirely possible that the LNPA is better able to detect one mechanism of initiation as compared to another. Another possibility is sensitivity, as on an absolute scale, drugs are all considered fairly weak sensitizers. It is quite possible that the sensitivity of the LNPA is limited, allowing detection of only the strongest of the pharmaceutical sensitizers. The issue of metabolic processing has been discussed above and remains a concern. It should be remembered that although this assay has had limited evaluation, no other assays for assessment of hypersensitivity potential of pharmaceuticals have been evaluated in a multilaboratory study such as this one. As a basis of comparison, the LLNA and the guinea pig assays for skin sensitization have been shown to accurately predict about 2/3 of the human skin sensitizers (Klecak et al., 1977; Klecak, 1985; Maurer, 1996).

In conclusion, the data show that the LNPA assay has promise for hazard identification of new drug candidates with the potential to cause systemic drug hypersensitivity in humans. The method as performed in this study showed reliable positive

responses for only a subset of drugs known to cause a high prevalence of human drug hypersensitivity. At this point, the LNPA could potentially be used for hazard identification at the point of screening a series of new drug candidates. A positive response might be interpreted as pointing to a discernable risk for human hypersensitivity. A negative response however would not provide absolute assurance that hypersensitivity will not be seen in clinical trials or post marketing usage. Results from the LNPA might be incorporated along with results of other studies and should not be evaluated in isolation from other preclinical or clinical trial data.

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