

Utility of historical vehicle-control data in the interpretation of the local lymph node assay

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The accepted approach to the interpretation of local lymph node assay (LLNA) data requires comparison of responses in the test groups with background activity found in concurrent vehicle-treated controls. However, of established value in the interpretation of toxicity test data is the use of historical control values that provide one criterion against which to judge the integrity of individual experiments. Specifically, the availability of robust and relevant historical control data permits examination of whether, in any individual experiment, control values fall within the expected range. With the most commonly used vehicle employed in the LLNA, acetone/olive oil (4:1) (v/v), the mean values, standard deviations and normal ranges are increasingly well established for a given laboratory, although there is some variation between laboratories, particularly with regard to expected ranges. Against this background, it is possible to identify (and, if appropriate, eliminate) a concurrent vehicle-control value that falls well outside the expected range. To explore critically the potential merits of this approach, one specific example is examined in detail.

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The local lymph node assay (LLNA) is a method for the predictive identification of chemicals that have the potential to cause skin sensitization. This assay has recently been adopted by the Organization for Economic Cooperation and Development (OECD, Guideline 429) (1). Adoption was predicated on a thorough and independent validation of the method in both the USA (2) and Europe (3). Full details of the validation data set have been published elsewhere (4). In OECD Guideline 429, the LLNA methodology is described in the context of hazard identification; that is, addressing the question of whether a chemical possesses the intrinsic potential to cause skin sensitization. In this method, chemicals are classified as skin sensitizers, if, at one or more test concentration, they are able to induce a 3-fold or greater increase in draining lymph node cell (LNC) proliferation compared with concurrent vehicle-treated controls (5, 6).

Although control values in the LLNA are relatively consistent, it became evident during inter-laboratory validation studies that, for a given vehicle, the most common being a 4:1 (v/v) mix-

ture of acetone/olive oil (AOO), the actual values varied somewhat between individual experiments as well as between laboratories. Examples of such variations have been published (7–10). Although there is no evidence that such variation has any impact on the accuracy or reliability of hazard identification, it is possible, in theory, that false-positive or false-negative results could arise from aberrant (either unexpectedly low or unexpectedly high) concurrent vehicle-treated control values. In addition, inappropriately high or low control data could also influence measurement of the relative potency of skin-sensitizing chemicals in the LLNA. Derivation of the estimated concentration of a chemical allergen, necessary to cause a 3-fold stimulation of proliferative activity in draining lymph nodes compared with concurrent vehicle-treated controls (the EC3 value), has been identified as a robust and valuable tool in risk assessment (6). Derived EC3 values have been shown to correlate closely with what is known of the sensitizing activity of contact allergens in humans and are increasingly being used for determination of relative potency as a first

step in the risk assessment process (11–16). Erroneous EC3 values could lead to an incorrect view of this important element of skin sensitization safety assessment (17).

It is important, therefore, to have mechanisms available to identify control values that, for whatever reason, fall well outside the normal range and that are, therefore, potentially suspect. In this paper, historical control values for AOO from 4 independent laboratories have been examined to provide an illustration of one way forward. To demonstrate how such information can be used practically with regard to the LLNA, a single case study is described.

Materials and Methods

Chemicals

3-Methyl pyruvate (99%) was obtained from Sigma (Poole, Dorset, UK).

Local lymph node assay

The assay was performed essentially as described elsewhere (1, 5). Briefly, groups of 4 CBA/Ca mice (7–12 weeks of age) were treated topically on the dorsum of both ears with 25 µl of test material or with an equal volume of the vehicle (AOO) alone. Treatment was performed daily for 3 consecutive days. 5 days following the initiation of exposure, all mice were injected via the tail vein with 250 µl of phosphate-buffered saline (PBS) containing 20 µCi of tritiated thymidine (Amersham International, Amersham, UK). Mice were sacrificed 5 h later, and the draining lymph nodes excised and pooled for each experimental group. Laboratory D used a slightly modified protocol involving groups of 5 CBA/J mice, with pairs of lymph nodes being processed from individual mice. A single cell suspension of LNCs was prepared by mechanical disaggregation. The LNC suspension was washed $\times 2$ in an excess of PBS and then precipitated with 5% trichloroacetic acid (TCA) at 4 celsius for 18 h. Pellets were resuspended in TCA, and the incorporation of tritiated thymidine measured by β -scintillation counting. A substance was classified as a skin sensitizer, if, at one or more test concentrations, it induced a 3-fold or greater increase in LNC proliferative activity compared with concurrent vehicle-treated controls. That is, sensitizing chemicals by definition elicit a stimulation index (SI) of 3 or more compared with vehicle controls.

In addition, the concentration of the chemical required to produce an SI of 3 (the EC3 value) was determined to provide a measure of relative skin-sensitizing potential. EC3 values were derived from

consideration of LLNA dose responses using a method described previously (11) that can be summarized briefly as follows. The EC3 value was calculated by interpolating between 2 points on the SI axis, one immediately above and one immediately below the SI value of 3. The vehicle-treated control value (by definition, SI = 1) cannot be used for the latter. Where the data points lying immediately above and below the SI value of 3 have the co-ordinates (a, b) and (c, d), respectively, then the EC3 value may be calculated using the equation:

$$EC3 = C + [3 - d/b - d](a - c)$$

Data analysis

For the control data set from each laboratory, a log normal distribution was fitted. From this, the mean of the distribution was derived, as well as the 2.5, 5, 95 and 97.5 percentiles to aid in the identification of potential outliers.

Laboratories

Laboratory A is Unilever Safety and Environmental Assurance Centre, Sharnbrook, UK; Laboratory B is RCC Ltd, Basel, Switzerland; Laboratory C is Syngenta Central Toxicology Laboratory, Macclesfield, UK; Laboratory D is Procter & Gamble, Human and Environmental Safety Division, Cincinnati, OH, USA.

Results

Test results derived from 3-methyl pyruvate, the substance whose data precipitated this report, are presented in Table 1. The initial study (test 1) showed a clear dose-response relationship, but in the context of an unusually low concurrent vehicle-control value of 134 disintegrations per minute (dpm)/node (for Laboratory B). This value was substantially below the mean control

Table 1. The results of local lymph node assay studies on 3-methyl pyruvate

	Results (dpm/node) (stimulation index)		
	Test 1	Test 2	Test 3
AOO control	134 (1.0)	477 (1.0)	467 (1.0)
1.0%	791 (5.9)	576 (1.2)	791 (1.7)
2.5%	1541 (5.11)	1531 (2.3)	1541 (3.3)
5.0%	2618 (5.19)	2232 (4.7)	2618 (5.6)
10%	4565 (34.0)	3791 (8.0)	4565 (8.9)
EC3 value (%)	<<1.0	2.4	2.4

Test 1 represents the original data, whereas test 2 represents the results from a repeat study with the same sample. Studies were carried out according to Organization for Economic Cooperation and Development (OECD) Guideline 429 (OECD, 2002). Test 3 shows the disintegrations per minute (dpm)/node data from test 1 reinterpreted with the mean control dpm/node data from laboratory B.

value obtained by this laboratory. Furthermore, it was considerably below the next closest value of 234 dpm/node. Although an EC3 value could not be derived from these data, it would clearly be less than 1.0%, and this was unexpectedly low (indicating a higher relative sensitization potency) in relation to what had been predicted from recent QSAR studies (18). Accordingly, the study was repeated in the same laboratory (test 2). Again, a dose-response relationship was apparent, but, on this occasion, the vehicle-control value of 477 dpm/node was much closer to usual experience, the mean value for this laboratory being 467 (Table 2). This latter test allowed derivation of an EC3 value of 2.4%; this being much closer to expectation. If the raw data from test 1 were reinterpreted using the historical mean control value derived from the distribution (Table 2) of 467, the derived EC3 value is also 2.4%. These data are presented as 'Test-3' in Table 1.

The historical control data for AOO vehicle were gathered from each of the 4 laboratories and are summarized in Table 2. A log normal distribution was the best fit to the data sets, and, from this distribution, mean values and percentiles were derived. The mean values were, as expected, not identical but were of similar order, varying between a low of 304 (Laboratory D) and a high of 467 dpm/node (Laboratory B). The ranges also showed some inter-laboratory variation, with the two laboratories (C and D) with the lowest mean values recording smaller ranges of control (spanning 600–700 dpm/node), whereas laboratories A and B had control values that spanned 800 and 1000 dpm/node, respectively. Both 2.5 and 5 percentiles were derived for each laboratory; it should be noted that due to the fact that the data have been fitted to a log normal distribution, these derived values may fall outside the observed range data, this being the situation for the 97.5 percentile values derived for 3 out of 4 of the laboratories.

In relation to the initial study on 3-methyl pyruvate, it can be seen that the original vehicle-control value of 134 dpm/node was below both

2.5 and 5 percentile values for laboratory B (187 and 216 dpm/node, respectively).

Discussion

The quality of experimental results is invariably dependent upon the relevance and robustness of the control data with which comparisons are made. This is of particular importance in the case of toxicological investigations that are employed for the purposes of hazard identification and safety assessment. In the case of the LLNA, concurrent vehicle-treated controls are the benchmark against which test data for a chemical are judged. This approach has proven to be valid for the purposes of regulatory hazard identification (1, 3, 4). It has also been shown to remain true where different vehicles have been employed, leading to differing levels of proliferation but always to a correct classification, for example in terms of EU regulations (19, 20). However, even in well-established procedures, biological experiments are not without occasional unexplained variations. In repeat dose toxicity studies, usually employing the rat, it has been recognized for many years that historical data are of potential importance for the interpretation of observations (21). This is particularly relevant for assessment of lifetime carcinogenicity studies, where robust information on the background incidence of spontaneously developing tumours is a cornerstone of accurate data interpretation (22, 23).

In the LLNA, the most commonly employed vehicle is AOO; it being the first on the list of recommended vehicles (1, 19). Examination of the data set contained in Table 2 shows that there is some variation in vehicle-control values within a single laboratory; furthermore, there exist differences between laboratories, inasmuch as the mean and ranges of control values for AOO show some variation. No doubt these reflect small differences in the background turnover of cells within the auricular (ear-draining) lymph nodes or are possibly due to changes in the efficiency of

Table 2. Historical control data for acetone/olive oil vehicle in the local lymph node assay for each of the four laboratories involved

Laboratory	N	Range (dpm/node)	Mean (dpm/node)	2.5/5 percentile	95/97.5 percentile
A	50	211–1000	410	220/243	692/765
B	23	134–1120	467	187/216	1010/1170
C	44	97–662	321	128/148	690/799
D	32	90–781	304	101/120	766/912

All studies were carried out according to Organization for Economic Cooperation and Development (OECD) Guideline 429 (OECD, 2002). Having fitted the range data to a log normal distribution, low (2.5 and 5.0) and high (95 and 97.5), percentile values have been derived.

scintillation counting. However, such variation need be of little moment for interpretation of data within a laboratory. Despite these variations, extensive inter-laboratory and intra-laboratory validations have demonstrated that the LLNA is a robust means of identifying chemicals that possess sensitization hazard (4, 6, 7). For example, in one laboratory over a period of 8 years, intra-laboratory differences in thymidine incorporation in lymph nodes derived from vehicle (AOO)-treated control animals of 159–496 dpm/node were recorded (7). The levels of thymidine incorporation for the test material, 25% hexyl cinnamic aldehyde (a recommended positive control for tests for skin sensitization) also varied with time, with levels of between 1639 and 4107 dpm/node observed. This inter-test variation, which was of little importance for the interpretation of the test data, is demonstrated by the fact that there was little variation in the SI values (range 7.2–13.9). In addition, it could be demonstrated that the inter-laboratory variation in mean and range of vehicle-treated control values had little effect on the interpretation of LLNA data, as those laboratories that recorded a relatively low vehicle-control value also recorded relatively low test values, resulting in all laboratories reporting similar SI values and EC3 values for this test substance (7).

An understanding of historical control values within an individual laboratory is a real asset in the interpretation of individual LLNA data sets, particularly in those instances when the vehicle-control value for a particular experiment appears unusually high or low. For this purpose, the example of 3-methyl pyruvate has been presented. In this specific case, a prior view of the probable relative sensitizing potency for this compound had been derived from an understanding of how this type of chemical causes skin sensitization (18, 24). Initial results (test 1) (Table 1) suggested skin-sensitizing potency well in excess of what was expected. However, it was clear that the vehicle-control value of 134 dpm/node was abnormally low compared with previous experience in this laboratory (B). For instance, this value was less than the 2.5 percentile value of 187 dpm/node, based on historical information. In comparison, use of the mean historical control value for AOO of 467 (Table 1) yielded an EC3 value of 2.4%. A repeat experiment with the same chemical in this laboratory yielded an EC3 value of 2.4% based upon a concurrent AOO control reading 477 dpm/node.

In summary, we have shown that historical vehicle-control data from the LLNA are a resource that can be deployed usefully in the

interpretation of individual data sets. In practice, a laboratory will need to accumulate a minimum of approximately 20 individual experiments with a particular vehicle system in order to generate a database of sufficient soundness for historical comparisons. One suggestion is that, using an evolving control data set, percentile values might be used as a basis for comparison. For instance, the guidance might be that any control value falling outside the 95 or 97.5 percentile might be viewed with some suspicion. However, this is only one of several practical approaches that could be used, and other strategies might prove equally or more effective. Irrespective of how this is used in practice, the important point being made here is that, in some instances where apparently unusual control values are obtained, it is wise to consider historical control values in reaching a judicious interpretation of LLNA data.

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