Assay of prick test inoculum volume. II. Average values and individual variability

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Introduction: The amount of reagent introduced into the skin by a prick test is critical in studies on the reproducibility and standardization of the method.

Objective: The purpose of the present study was to measure the average volume and the individual variability of the inoculum performed with a prick test standardized technique.

Methods: Two hundred forty prick tests—16 inoculations per subject—were performed on the volar sides of arms of 15 healthy volunteers. The tests were performed by a skilled tester whose technical performance complied with the criteria of good reproducibility described in international guidelines. A 1-mm tip standard device and a 50% glycerosaline solution labeled with Tc99m were used for the test. The inoculum size was calculated using a direct assay method based on the gamma camera.

Results: The average volume of the prick test inoculum was equal to 0.016 microliters, with a remarkable dispersion of the values around the mean (median, 15906; range, 418 to 82253 picoliters). Further, we observed great variability from one subject to another and great variability in the same subject from one skin site to another. A statistical analysis of the data shows that this variability depends on the individual characteristics of the subjects examined. A skilled tester using a standardized technique is not responsible for significant variability.

Conclusions: Even when performed by a skilled operator and with standardized techniques, the prick test shows great limits of reproducibility, at least as far as the size of the inoculum volume is concerned. The variability of the inoculum depends, in a statistically significant way, on the subject's individual characteristics and therefore can be reduced only within certain limits by the standardization and perfectibility of the technique.

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INTRODUCTION

The skin prick test is one of the most widely used techniques to diagnose allergies. Its undeniable advantages are safety and rapidity of performance. Yet a number of older studies have shown poor reproducibility when using this method.[1–8]

Various factors affecting the reproducibility of the prick test can schematically be related to causes depending on:

- individual biologic responsiveness;
- the tester and the technique used to perform the test;
- reagents; and
- the methods used to delimit and measure skin reactions.[2,3,7,9–11]

Variability factors depending on individual responsiveness obviously cannot be eliminated or standardized. A number of studies have demonstrated that the main cause of variability is almost certainly due to the amount of allergen or other biologic reagent inoculated by pricking the skin, ie, the size of the inoculum.[1–7,9,11] The performance of the prick test a priori implies a high probability of significant variations in the volume inoculated per test, as its size depends on the tester's manual skill and on the technique used to perform the skin test.

We began to address this problem by standardizing the method. First we conformed testing procedures (as suggested by Pepys)[12] and then used standard precision devices (Morrow-Brown needles and lancets).[13] By standard precision devices we mean a device the dimension and shape of which could always ensure the same penetration into the skin and consequently the intradermal transport of the same amount of reagent. Standardized precision devices are widely used today and several types are available on the market.

Several studies show that the use of standard needles improves the reproducibility of the prick test. Nevertheless, the most important factor in obtaining high reproducibility seems to be the technical performance of the test, according to the experience and the skill of the tester.[14]

Several problems arise when verifying these methodologic assumptions experimentally with direct measurements of the volume inoculated into the skin. To the best of our knowledge the only calculation of the average volume inoculated was indirectly performed by Squire about 50 years ago, on the basis of the dose-response correlation to histamine.[15]

A previous report of ours on the subject,[16] showed great methodologic limits. The limits mainly concerned the experimental proofs of the reliability of the assay method. So new investigations were necessary.

In the present study we made direct, precise measurements of the prick test volumes to assess their average size

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Table 1. Result of the Assay of the Average Inoculum Volume by Prick Testing in the Individual Subjects Examined in the Study

N	Count	Average	Variance	SD	Median	Minimum	Maximum
1	16	764.25	59708.7	244.354	717	428	1227
2	16	3494	2.21697×10^6	1488.95	3427.5	1198	6342
3	16	1219.13	348727	590.531	1076.5	418	2179
4	16	1626.38	110404	332.27	1560	1231	2445
5	16	10272.1	3.29441×10^7	5739.69	9020.5	4241	24490
6	16	35492.9	1.1906×10^7	10911.4	33518	22830	56611
7	16	22992.6	5.92725×10^8	7698.86	25086	13202	34812
8	16	37156.1	2.18146×10^8	14769.8	36332	14800	65497
9	16	42097.9	4.04333×10^8	20108	36279	14610	82253
10	16	26119.3	1.14903×10^8	10719.3	22415	14582	44681
11	16	29277.4	1.32228×10^8	11499.1	25437.5	16226	60284
12	16	17076.7	1.77853×10^7	4217.26	17315.5	11073	28845
13	16	13744.2	9.88551×10^7	9942.59	8798.5	5386	37183
14	16	8631	5.17007×10^6	2273.78	8579.5	5831	13547
15	16	20895.9	1.74444×10^7	4176.65	19847.5	16430	30555

and the degree of individual variability. The tests were performed with a standardized technique by a skilled tester, ie, an operator whose technical performance complied with the criteria of good reproducibility outlined in international guidelines.

METHODS

The study comprised a group of 15 healthy volunteers (9 men and 6 women, aged 59 to 68 years, average age 64 ± 4), who had given their consent.

Before performing any tests, the tester checked the correct reproducibility of his own technique in conformity with Aas and Belin's protocol and with the criteria recommended by the EAACI.[14,17]

The prick tests were performed on the volar sides of the subjects' forearms, with a 1-mm tip standard Morrow-Brown needle (a disposable puncturing device in acrylic copolymer with pyramidal tip, produced by Laboratorio Farmaceutico Lofarma, Milan, Italy). We used this device because we had obtained a high degree of reproducibility in in vivo tests in a previous study.[18]

We performed a total of 16 tests on each subject, 8 on each forearm, placed in double file. For all 240 inoculations we used a 50% glycerosaline solution, routinely used as diluent in allergy work, labeled with Tc99m.

We demonstrated a high degree of precision, reliability, and safety of this method in in vivo studies in Part I of the study.[19a]

The choice of elderly volunteers was, however, another prudent step. Tissue sensitivity to radiations varies according to exposure intensity and length and to tissue characteristics (highest for gonads and tissues whose cells are at the stage of active reproduction). The younger the subject's age, the higher the harmful effects of radioexposure.[19]

The average dose absorbed by a tissue or organ, weighted for the quality of the radiation (equivalent dose) and that absorbed by the whole body (effective dose) is expressed in "Sievert" (Sv).[20] The effective dose is equal to the sum of the equivalent doses in all tissues and organs of the body weighted for the tissue effects of radiation.

According to current laws and to the recommendations of the International Commission on Radiological Protection, the whole body dose should not exceed 1 mSv/per year.[21]

The tests were acquired and recorded by an automatic computer programme. The statistical analysis of the data was carried out with the software Statgraphic Plus 3.0 (Manugistic Inc, Rockville, Maryland, USA).

RESULTS

The results of the assay of the average volumes inoculated into each patient are recorded in Table 1.

The data show great differences in average volume values, and in the same subject from one skin site to another, without any definite order (Table 2). Further, in each subject we can find a remarkable dispersion of the values with variation coefficients sometimes over 50% when compared with the mean.

The radioactivity absorbed (in terms of equivalent dose and effective whole body dose) was 1,000 to 10,000 times lower than in a common scintigraphic exam (for instance a thyroid scintigraphy). Our method can therefore be used safely without any problems with any subject, even infants, provided ethical rules and clinical appropriateness are respected.

If we consider the data as a whole to determine the inoculum average assay in the subjects examined, we notice a non-gaussian distribution (Fig 1). The frequency curve of the sampled values

Table 2. Example of the Distribution of the Inoculum Volumes (picoliters) in One of the Subjects (from top downwards, from the cubital fold to the wrist)

Right Forearm		Left Forearm	
Radial Side	Ulnar Side	Ulnar Side	Radial Side
34812	28801	28646	15298
25748	24973	25859	15587
34681	25199	13202	13202
18723	32057	13897	17197

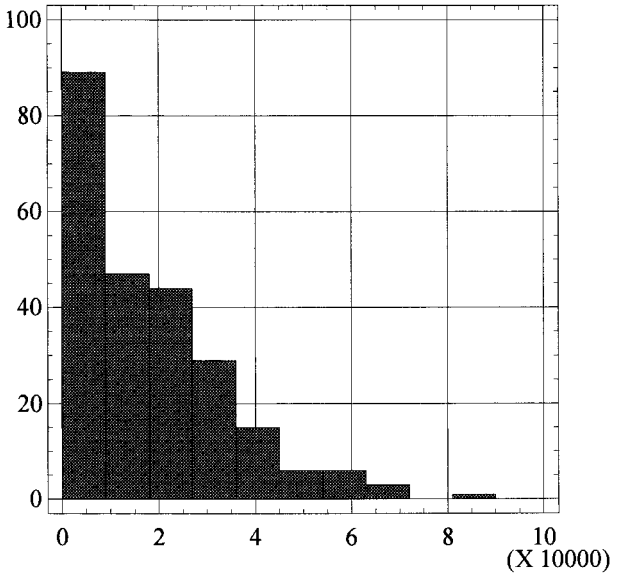

Figure 1. Histogram of the frequency distribution of the assays in picoliters (cf. report).

takes on a bell shape, deformed by a long tail on the right, and skewed (by a concentration of the values) on the left. The Kolmogorov and Smirnov test (overall statistic DN = 0.135947; *P* value = .000281) and the Shapiro Wilks test (W statistic = 0.885095; *P* value = 0) confirm a significant spread from normality.

This distribution is quite common in biologic assays of very small volumes, and is known as the law of proportional effect: Galton-Mac Allister's Law.[22] It probably demonstrates that variations in the size of the inoculum are directly proportional to the very size of the inoculum. That is, the higher the volume of the inoculum, the more significant the effects of variability factors.

Contrary to expectations, a logarithmical transformation of the data does not show a normal distribution, but a bimodal distribution. We therefore recognize two different populations, respectively, with either a low or a high degree of skin penetration of the reagent.

The values of the medians of each of the 15 subjects taken separately are normally distributed (Kolmogorov-Smirnov test: overall Statistic DN = 0.188813, *P* value = .658745; Shapiro Wilks test: W Statistic = 0.909334, *P* value = .101883, NS).

In the group of subjects examined, the average value of the inoculum was about 0.016 microliters (median = 15906 picoliters; range: 418 to 82252 picoliters).

A variance analysis of the data, however, demonstrates that the variability we noticed depends in a statistically significant way on the individual characteristics of the subjects examined. A tester using a standardized technique is not a significant source of variability (Table 3).

DISCUSSION

In the diagnosis of allergic diseases, a skin test can be likened to a specific challenge test, the practical purpose of which is to prove a biologic reaction to a certain allergen. In clinical practice, the skin test shows good reproducibility and a high degree of diagnostic reliability, whatever device may be used.[11,23,24] Of course, this is provided the test is performed by a skilled operator whose technical performance complies with the criteria provided for by international guidelines.

A quantitative test interpretation (degree of reactivity and threshold value) is based on the dose-response correlation. This implies the possibility of precisely determining (and varying) the size of the stimulus.

From this point of view, the prick test should be regarded as a qualitative test. Unlike an intradermal reaction, when the prick test method is used one cannot predetermine the challenge

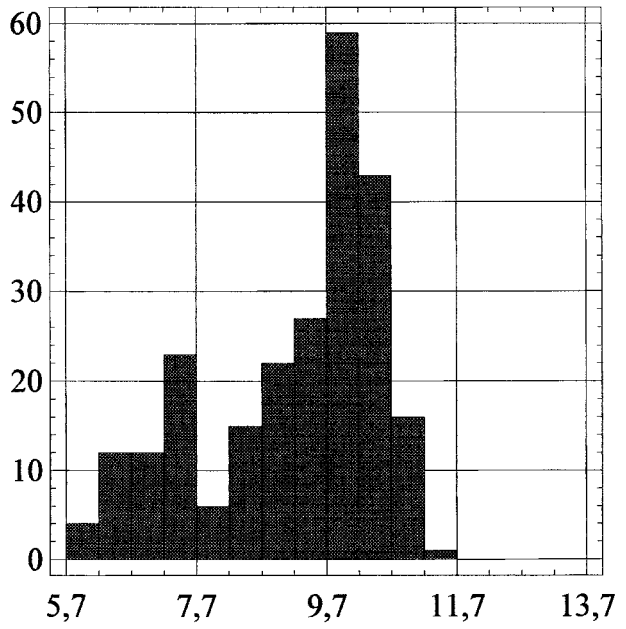

Figure 2. Histogram of the frequency distribution of the assays in log-volumes (cf. report).

Table 3. Variance Components Analysis and Kruskal-Wallis Test*

Variance Components Analysis						Kruskal-Wallis Test	
Source	Sum of Square	Df	Mean Square	Var. Comp.	Percent	Test Statistic	*P* Value
Total (corr.)	6.16021×10^{10}	239					
Patients	4.3259×10^{10}	14	3.08993×10^{9}	1.88025×10^{8}	69.76	199.783	.0
Repetitions	1.83432×10^{10}	225	8.15251×10^{7}	8.15251×10^{7}	30.24	2.23872	.9994

* The main cause of the variability of inoculum volumes by prick testing lies in the subjects examined. The rerunning of the tests contributes, in this series, to about 30% of the general variability. Kruskal-Wallis test shows that the variance observed depends on the individual differences of the subjects examined. A skilled tester, instead, is not a statistically significant source of variability.

dose. Alternatively, one can vary the concentration of diagnostic extracts but not the volume inoculated, which can only be measured after the performance of the test. This obviously presents several problems. The measurement of the inoculum volume is critical in any quantitative interpretation of the prick test and in the studies of the standardization and reproducibility of the method.

As stated in the introduction, performing the prick test a priori implies a high probability of great variations in the volume inoculated per test. The standardization of the method has been a way of solving the problem. In the past, indirect measurements of the inoculum were taken in order to verify whether this objective (equal volumes per prick test) had been attained. These measurements were based on the calculation of the dose-response correlation to the allergen or to other skin reagents.

Such estimates, however, are liable to approximations and evident methodologic limits. The limits mainly result from the imprecise method of delimiting and measuring skin responses,[10,11,25,26] and from possible non-linear progression of the dose-response correlation curve.[15,27–30]

In the present study, we made direct measurements of the inoculum volume with great precision and accuracy. These measurements were not affected by variability factors stemming from biologic reactivity of the skin. The experimental results of this study are different, from many points of view, from the conclusions and data so far accepted in literature.

Almost half a century ago, Squire[15] obtained a quantitative estimate of the average volume of the inoculum by comparing, in the same subjects, the skin responses observed by prick tests to the ones challenged by known amounts of histamine inoculated intradermally with precision needles. This experiment enabled him to calculate an inoculum volume on average equal to 0.003 microliters. Our estimates gave a value five times higher than Squire's, with great differences among the various subjects and with a very wide range of variability.

If we analyze the data, this variability seems to depend almost exclusively on the individual characteristics of the subjects examined. A skilled tester using standardized techniques is not a statistically significant source of variability.

We also observed great differences in the inoculum volume per test in the same subject, from one skin site to another, without any precise order. In previous studies it had been noticed that, in the same subject, the intensity of skin responses to the same dose of reagent is higher in ulnar sites than in radial ones and progressively decreases from cubital sites towards the wrist.[9] In terms of inoculated volumes, the dispersion of values appears quite random and therefore cannot be correlated to the results of such studies.

Since the solutions used for tests (similar to the negative control used in allergy practice), do not result in any skin responses, the variability factors affecting the size of the inoculum are certainly different and independent of individual biologic reactivity. They are therefore likely to depend on skin characteristics, the type of the skin in general, its degree of trophism and hydration and, above all, the integrity of epidermal layers.

For this reason the choice of elderly subjects may have increased the degree of variability of the inoculum by prick testing.

It is common knowledge that serious errors in performing the prick test result in mistakes in the interpretation of skin tests. False positives can be caused by scarification, while false negatives can be due to over-superficial injections with insufficient penetration of the allergen. The findings reported in this study confirm that it is necessary to perform the prick test in a standardized way. Testers should check the reproducibility of their technique very carefully, as recommended by various scientific associations.

In the case of a well-standardized performance of the prick test, the variability of the skin response has always been thought to depend, in general, on the biologic components of skin reactions rather than on significant variations in the amount of allergen inoculated into the skin. The findings of the present study, on the other hand, show that the individual characteristics of the subject's skin can cause great variations in the inoculum volume.

Such variations in the inoculum volume are very likely to be a significant variability factor in skin reactions. Variability factors like biologic components of the skin reactions, can be neither eliminated nor reduced beyond a certain limit by the standardization of prick testing methods and techniques.

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