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Technique for Differentiating Particles That Are Cell-Associated or Ingested by Macrophages

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Phagocytosis is a two-step process involving attachment and ingestion of particulate material. It is often difficult to determine under a light microscope whether the particles are actually ingested or are merely attached to the cell. A more accurate, easy to perform technique with the use of xylene has been developed for determining the difference between the attachment and ingestion of polystyrene latex spheres. The xylene treatment dissolves the extracellular spheres, leaving only the intracellular spheres to be counted by the experimenter to obtain a more accurate phagocytic index. This technique also allows an investigator to get an ingestion index, an attachment index, or both.

Phagocytosis is a step-by-step process involving attachment of the particle to the cytoplasmic membrane, invagination of the membrane, and the formation of a phagocytic vacuole consisting of the particle surrounded by the cytoplasmic membrane (2). With increasing interest in the function of phagocytic cells, it is of prime importance that a reliable method of accurately measuring phagocytic efficiency be available. When evaluating phagocytic indices, it is often difficult to determine under a light microscope whether particles are actually ingested or are merely attached to the cell. A particle on the surface of the cell may appear to be ingested when it is only adhering to it. This leads to errors in evaluating ingestion during phagocytosis. A more accurate technique for determining the difference between attachment and ingestion of latex spheres has been developed.

MATERIALS AND METHODS

Alveolar macrophages were harvested by the pulmonary lavage technique from New Zealand rabbits of both sexes weighing between 1 and 2 kg (1). The lung effluent was centrifuged at 365 × *g* for 15 min at 5 C, and the resulting cell button was suspended in 2.65 ml of medium 199, Hanks base (BBL), with 20% heat-inactivated rabbit serum. The cell suspension was placed in a 25-ml siliconized Erlenmeyer flask. To the macrophage suspension, 0.35 ml of polystyrene latex particles, 1.001 μm in diameter (Dow Chemical Co.), in a physiological saline solution was added. At this time, there were 10.3×10^6 macrophages/ml, giving a ratio of 1 macrophage to 17.5 latex spheres. The flask was then placed in a reciprocating-shaker bath at 82 rpm at 37 C.

After a 30-min incubation, approximately 0.02 ml of the cell suspension was smeared onto a glass slide by use of a Pasteur pipette. The smears were fixed in a Formalin vapor, stained with Giemsa stain, decolorized with 10% rosin solution (wt/vol in ethanol), and rinsed with ethyl alcohol. The phagocytic index is generally defined as the number of cells that have latex spheres that are either touching the plasma membrane or are inside the cells. The phagocytic index was determined by counting 600 macrophages on approximately two-thirds of the area on each of four slides. Macrophages were classified on the basis of numbers of spheres phagocytized: 0, 1 to 3, 4 to 6, 7 to 9, or 10 or more. The slides were then allowed to sit in 100% xylene for 3 h with occasional shaking and were recounted according to the same method of classification.

RESULTS

Data from these experiments indicate that a more reliable index of phagocytosis results when slides are treated with xylene after fixation and staining. This method clarifies the ambiguity of whether spheres are in or out by dissolving those which are merely in juxtaposition, while permitting those which have actually been phagocytized to persist as clear vacuoles within the cytoplasm. This is illustrated by the difference between Fig. 1a, which shows a field untreated by xylene, and Fig. 1b, which shows the same field after xylene treatment.

Statistical analyses were used to determine whether xylene treatment results in more accu-

rate phagocytic indices. Table 1 shows a representative sample of the raw data for several individual slides and the pooled data for those slides. For each slide, the homogeneity of the number of latex spheres phagocytized before and after xylene treatment was tested by a contingency table analysis. The results of these analyses show that the number of latex spheres *observed* to have been phagocytized prior to xylene treatment differs significantly ($P < 0.0001$) from the number counted after treatment with xylene.

The differences in the proportions of before to after xylene treatment were not consistent among the slides. For example, observe from Table 1 that the number of cells judged to have phagocytized no latex spheres varied from 368 to 454 for the before xylene treatment counting, whereas the number varied from 532 to 578 for the xylene treatment counting. This is indicated by the statistically significant chi-square of 34.03 ($P < 0.001$). The greater variability of the results for the before xylene treatment counting was the main cause of the rejection of the test for homogeneity.

It was hypothesized that the thickness of the smear caused this significant heterogeneity. To ascertain whether or not the thickness of the smear on the glass slide could cause increased variability before xylene treatment, the following experiment was performed.

A cell sample was placed on the left side of

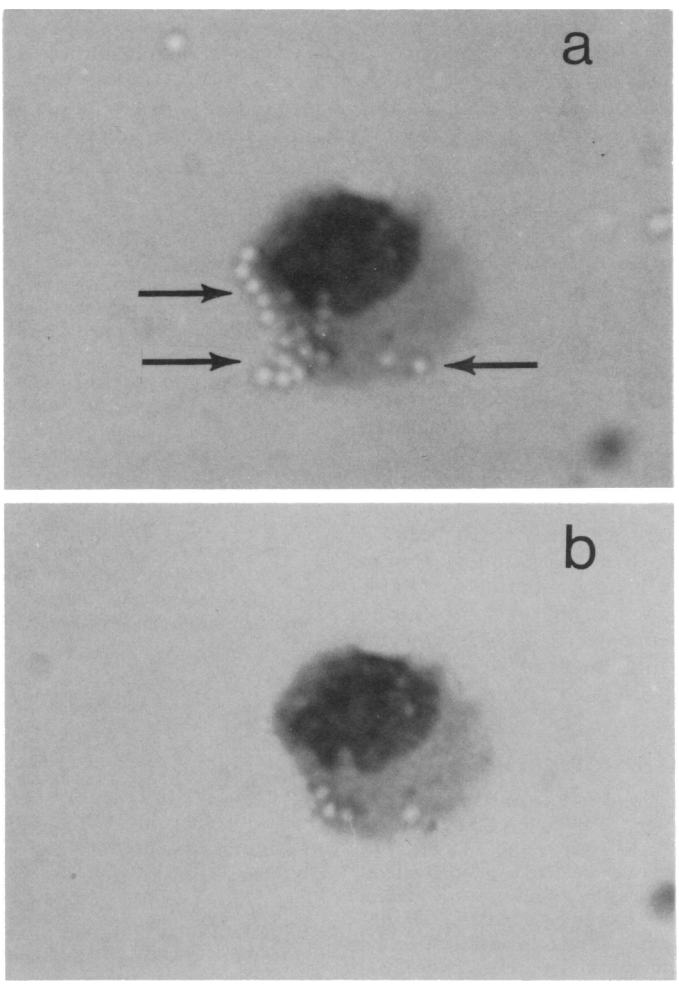

FIG. 1. *Photomicrograph of the phagocytosis of polystyrene latex spheres by alveolar macrophages. (a) Before xylene; (b) after xylene. The arrows point to the extracellular spheres that were removed by xylene treatment.*

TABLE 1. *Macrophages grouped according to the number of phagocytized latex spheres*

Slide	Treatment	No. of macrophases for indicated no. of latex spheres phagocytized				
		0	1-3	4-6	7-9	≥10
1	Before xylene	414	129	14	4	39
	After xylene	551	13	8	4	24
2	Before xylene	368	168	27	6	31
	After xylene	532	24	10	1	33
3	Before xylene	454	92	12	7	35
	After xylene	540	22	9	1	28
4	Before xylene	374	162	18	8	38
	After xylene	578	4	0	1	17
Pooled	Before xylene	1,610	551	71	25	143
	After xylene	2,201	63	27	7	102

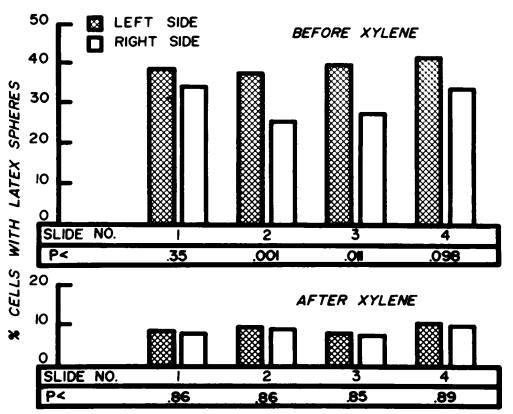

FIG. 2. *Effect of smear thickness on phagocytic index. Note the difference in phagocytic index between the left (thick) and right (thin) sides before xylene treatment. This variation is eliminated by xylene treatment.*

the slide and smeared to the right. Thus, the thickness of the smear decreased from left to right. Two hundred cells were observed in approximately a 0.6-cm interval at each end of four newly prepared slides. The cells were classified as having no latex spheres or having one or more spheres, before and after xylene treatment. This time only two categories of classification were used because it was found that, in all cases when five classes were used, the first two categories accounted for more than 85% of the total chi-square value.

Figure 2 shows the data obtained from the four slides. Before treatment with xylene, there was a statistically significant difference between the results for the left side versus the right side for slides 2, 3, and 4. After xylene treatment, however, counts obtained from the left side were not statistically different from counts obtained from the right side. The experimental results support the hypothesis that smear thickness is a factor enhancing the variability of the results for the before xylene counting. This variability is corrected by the xylene treatment.

TABLE 2. *Margin of error with an overall risk of α = 0.05*

Sample estimate of percent phagocytosis[a]	Margin of error (%) for indicated number of cells counted					
	100	200	300	400	500	600
1	1.95	1.38	1.13	0.98	0.87	0.80
2	2.74	1.94	1.58	1.37	1.23	1.12
3	3.34	2.36	1.93	1.67	1.50	3.16
4	3.48	2.72	2.22	1.92	1.72	1.57
5	4.27	3.02	2.47	2.14	1.91	1.74
6	4.65	3.29	2.69	2.32	2.08	1.90
7	5.00	3.54	2.89	2.50	2.24	2.04
8	5.32	3.76	3.07	2.66	2.38	2.17
9	5.61	3.97	3.24	2.80	2.51	2.29
10	5.88	4.16	3.39	2.94	2.63	2.40
15	7.00	4.95	4.04	3.50	3.13	2.86
20	7.84	5.54	4.53	3.92	3.51	3.20
25	8.49	6.00	4.90	4.24	3.80	3.46
30	8.98	6.35	5.19	4.49	4.02	3.67
35	9.35	6.61	5.40	4.67	4.18	3.82
40	9.60	6.79	5.54	4.80	4.29	3.92
45	9.75	6.89	5.63	4.88	4.36	3.98
50	9.80	6.93	5.66	4.90	4.38	4.00

[a] Phagocytic index.

When determining ingestion indices after xylene treatment, it is not always necessary to count 600 cells. Table 2 shows the margin of error that can be expected for various combinations of phagocytic index and number of cells counted. For example, in the xylene treatment data presented in Table 1, which had a phagocytic index of 10%, there was a 2.40% margin of error when 600 cells were counted. If only 200 cells had been counted, the margin of error would have been 4.16%. Therefore, depending on the amount of error acceptable, fewer cells can be counted.

It was hypothesized that other factors might influence this model. Additional experiments were designed to test the efficacy of xylene treatment with the following different parameters: (i) suspending cells in media containing various concentrations of heat-inactivated rab-

bit serum (0, 5, 10, and 20%), (ii) shaking in xylene for 10, 15, and 20 min versus sitting for 4 h, and (iii) washing the cells three times with saline versus the xylene treatment in combination with the above parameters. The experiment was done as previously described except that the above conditions were tested. Four hundred cells per slide were counted.

Two-by-two contingency table analyses of these data were performed to test the effect of these parameters. There was no statistical difference ($P < 0.25$) among the results after shaking in xylene for the various time periods studied. This is important when the possibility that xylene dissolves intracellular spheres is considered. The data also indicate that, as compared with allowing them to sit for 4 h, shaking the slides increased the accuracy of the technique by removing more extracellular spheres over any given time period, except when 20% serum was used. In this case, there was no statistical difference between shaking and sitting in xylene.

The data support the hypothesis that only extracellular particles are removed since xylene treatment of the slide with subsequent shaking for 10 or 20 min or sitting for 240 min as compared with xylene treatment of the slide with shaking for 15 min yielded no significant statistical differences ($P < 70.25$). Figure 3 illustrates that the number of intracellular spheres did not significantly differ after 10 min of xylene treatment, even though the extracellular particles had been rapidly reduced. The stability of the ingested particles thus makes this a reliable technique for phagocytic studies. Contingency table analyses also showed that, although washing successfully removed many extracellular particles, it was not as effective as the xylene model.

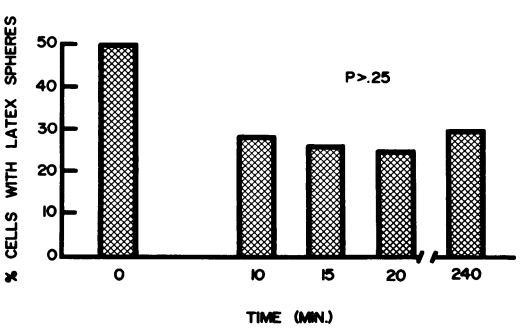

FIG. 3. *Effect of xylene on number of intracellular latex spheres with time. The P value indicates that further reduction in percent of cells with latex spheres does not occur with increased time in xylene.*

DISCUSSION

The data presented show that more accurate ingestion indices are obtainable when xylene treatment is used. This method ensures that, for ingestion indices, slight variation in smear thickness will not cause significant counting differences, and therefore fewer cells on a small area of the slide can be counted.

This technique can also be used to study the attachment phases of phagocytosis. When the phagocytic index is determined before and after xylene treatment, the difference between the two counts is the attachment index. However, when investigating attachment, more care must be taken. As previously discussed, slide thickness makes a difference in the before xylene treatment count. Therefore, the slides must have identical amounts of fluid smeared evenly, and large areas of the slide must be counted.

For these experiments, a large number of latex spheres were used, which sometimes caused a clumping of spheres on some macrophages. Under these conditions, 3 to 4 h of xylene treatment did not remove them completely. However, the xylene did partially dissolve them as evidenced by a different light refraction effect which was easily noticeable through a microscope.

The techniques which have been used for distinguishing true phagocytosis from cell attachment are often complicated and sometimes unsatisfactory. Rabinovitch (3) and Smith and Goldman (4) have also described a technique for differentiating intracellular and extracellular particles during phagocytosis. Rabinovitch used treated horse erythrocytes which had a yellowish hue when ingested by peritoneal macrophages. Extracellular erythrocytes were brownish-purple. Smith and Goldman studied phagocytosis of neutral-red dyed, heat-killed *Candida albicans* cells by stimulated peritoneal macrophages. Intracellular *Candida* cells were red. Concanavalin A, which is used for stimulation, enhances the red color of intracellular particles. The functional state of the macrophage also affects the change of intracellular *Candida* to a red color. If polystyrene latex spheres can be used for an experiment, then the xylene technique is less complicated and time-consuming than the above-mentioned methods. In addition, the xylene technique can be used on unstimulated cells, and, although the functional state of the macro-

phages may affect the phagocytic index, it will not have an effect on the xylene technique itself.

The xylene technique is reliable and easy to perform and can be adapted to numerous applications. Although this technique fails to aid those whose research demands the use of living organisms, it does offer a more sensitive procedure for others whose studies demand an accurate index of phagocytosis.

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