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Measurement of inflammatory reactions by a dye dilution method

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The rat pleural cavity has been shown to be useful for studying inflammatory reactions (Hurley & Spector 1965; Hurley et al 1966; Di Rosa et al 1971; Capasso et al 1975; Yamamoto et al 1975) and assessing the effects of anti-inflammatory agents (Sancilio 1969; Vinegar et al 1973; Tarayre et al 1979).

We describe a dye-dilution technique for measuring inflammatory reactions elicited in the rat pleural cavity. Exudate volume was determined from the decrease in absorbance of a dye solution placed in the cavity, thereby avoiding the need for quantitative sample recovery. Compared to a direct sampling technique, this method is more accurate and precise for quantitating both exudate volume and cellular influx.

The simplicity and accuracy of the technique suggests its potential utility for other animal models requiring quantitation of body cavity fluid and cellular contents.

Materials and methods

Preparation of dye solution. Ethylenediaminetetraacetic acid, tripotassium salt (EDTA) (Eastman Kodak Co., Rochester, N.Y.) 5 g was dissolved in 100 ml of 0·9% NaCl (saline) (Abbott Laboratories, North Chicago, IL) and phenol red (Mallinckrodt Chemical Co., St Louis, MO), 325 mg, added together with saline to 1 litre. A 0·072 M phosphate buffer of tribasic sodium phosphate, $Na_3PO_4.12H_2O$, (Mallinkrodt Chemical Company, St Louis, MO) was used to dilute the phenol red-exudate samples and make them alkaline.

Dye-dilution technique. Male, Sprague-Dawley rats (King Animal Supply Laboratories, Inc., Oregon, WI), 160–200 g were killed with carbon dioxide and 2 ml of phenol red solution was added to the pleural cavity and thoroughly mixed with the fluid contents of the cavity which were then transferred to a plastic test tube. A 50 μl sample was withdrawn from each tube and mixed with 10 ml of ISOTON II diluent (Coulter Electronics, Inc., Hialeah, FL). The exudate cells were counted electronically after the addition of several drops of ZAPOGLOBIN (Coulter Electronics, Inc., Hialeah, FL) using a Coulter model FN counter. The tubes containing the exudate-phenol red mixture were centrifuged at 750 *g* for 15 min to sediment the cells and 100 μl aliquots of the resulting supernatants were diluted with 3·9 ml of phosphate buffer and the absorbances of the samples measured at 560 nm.

* Correspodence.

The exudate volume was calculated from the absorbances of the samples according to the equation:

$$V_1 = \frac{V_2 (A_2 - A_3)}{(A_3 - A_1)}$$

where: V_1 = Unknown volume to be determined (exudate), V_2 = Volume of dye added to cavity (2 ml), A_1 = Absorbance of exudate, A_2 = Absorbance of phenol red solution, A_3 = Absorbance of exudate and phenol red mixture.

The absorbance of diluted (1:40) exudate fluid at 560 nm (A_1) is low and for many applications can be assumed to be zero. (The average absorbance of a pooled diluted carrageenan-induced rat pleural exudate was 0·003 absorbance units).

Direct sampling technique. The fluid contents of the cavity were withdrawn using a siliconized pipette. The cavity was washed with 3 ml of saline containing 5 mg of EDTA and the volumes measured in siliconized glass graduated centrifuge tubes. The numbers of cells in the exudate and wash fluids were counted electronically.

Exudate simulation. Oyster glycogen (Sigma Chemical Company, St Louis, MO) (12 ml, 1% in saline) was injected into the rat peritoneal cavity. Elicited cells were collected 18 h later and suspended in isotonic phosphate buffered saline (pH 7·0) containing 0·15% EDTA to give 44×10^6 cells ml^{-1}). This cell suspension was used to simulate an inflammatory exudate. This technique was used to make a more exact comparison of the two sampling methods by eliminating the variance associated with differences between animals when exudates are induced by an inflammatory stimulus. Various volumes were added to the pleural cavity and the volume and number of cells in the cavity were determined by either the dye-dilution or direct sampling technique.

Carrageenan pleural exudate. A pleural carrageenan exudate was elicited by injecting 0·2 ml of a 1·25% carrageenan solution (Viscarin, Marine Colloids, Bar Harbor, ME) into the pleural cavity and collecting the effusion 4 h after challenge. The exudate from the pleural cavities of 24 rats was pooled and centrifuged at 750 *g* for 15 min before use.

Results

The linearity of the change in absorbance produced by a relatively broad range of exudate volumes was evaluated.

A pooled carrageenan-induced pleural exudate, which had been centrifuged to sediment cellular contents, was used. Volumes of this exudate from 0·05 ml to 1·6 ml were added to test tubes followed by 2 ml of phenol red solution. The reduction in absorbance of the dye solution was then plotted against the dilution produced by the added exudate fluid. These results when fitted by a least squares regression technique showed excellent linearity over the range of volumes tested (correlation coefficient >0·999).

Table 1 gives the results of the comparison of the dye dilution with the direct sampling method. With the dye-dilution method, the recovery of added fluid and cells is greater as well as less variable.

Discussion

The dye-dilution technique provides a useful and reliable method for quantitating inflammatory exudates elicited in the pleural space. It has several advantages that make it more rapid and easy to perform than the direct sampling technique. After the exudate and dye are mixed thoroughly, only a sample of adequate volume for spectrophotometric reading and a cell count need be removed from the cavity. The direct sampling method requires that an attempt be made to remove all of the exudate in order to measure the volume. Because residual exudate fluid cannot be recovered from the walls of the cavity, the dye-dilution technique is inherently a more sensitive method for measuring small exudate volumes such as occur in drug inhibition studies. In addition, because a relatively large volume of dye solution (2 ml) is added to the cavity, the need for additional washings required by the direct sampling method to insure good cell recovery, is precluded.

With the dye-dilution method, the viability of the inflammatory cells from the rat pleural cavity following carrageenan challenge has been examined and found to be greater than 99% (measured by trypan blue exclusion). Microscopic examination of exudates collected by both methods from a 4 h carrageenan pleural reaction showed no significant differences between types of cells recovered with polymorphonuclear leukocytes being predominant (>90%).

Potential disadvantages of the dye dilution technique are the dilution of the exudate and the presence of phenol red in exudate samples, both of which may compromise additional biochemical studies. We have found that protein determinations, which are commonly of interest in inflammatory studies, can be performed in the presence of phenol red using the method of Bradford (1976). In those instances where the dilution or dye pose problems, selection of a smaller volume of dye or a different dye (or substituting a fluorescent or radioactive marker for the colorimetric marker) may overcome these drawbacks.

Implanted sponge models have also been used to study inflammatory reactions (Wiener et al 1973; Higgs et al 1976) as they allow the quantitation of both exudate and cellular accumulation. The use of a dye-dilution technique to measure sponge fluid contents may offer some advantages over existing techniques. The simplicity of the technique suggests its potential applicability to many studies which involve the measurement of fluid or cellular contents.

Table 1. Comparison of dye-dilution and direct sampling methods. Glycogen-induced rat peritoneal exudate cells suspended in phosphate buffered saline (44×10^6 ml^{-1}) were injected at various volumes into the rat pleural cavities (n = 8). The volume and number of cells added to the cavities were determined by either the dye-dilution or direct sampling technique. The average fluid and cellular contents of normal rat pleural cavities were determined by each method and subtracted from the appropriate volume and cell number measurements.

	Exudate volume determinations			
	Dye-dilution		Direct sampling	
Vol. added to pleural cavity (ml)	Vol. recovery (%)	Coeff. var. (%)	Vol. recovery (%)	Coeff. var. (%)
0·2	106·0	8·4	81·3	21·8
0·4	102·7	5·9	87·6	20·2
0·8	104·8	5·0	90·7	10·4

	Exudate cell number determinations			
	Dye-dilution		Direct sampling	
Av. no. cells added to cavity ($\times 10^6$)	Cell recovery (%)	Coeff. var. (%)	Cell recovery (%)	Coeff. var. (%)
9	107·1	11·5	85·0	28·3
17	93·3	10·7	88·0	22·7
35	92·4	8·2	75·1	12·3

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