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PAPER

QUANTITATIVE EVALUATION OF MOUSE PERITONEAL MACROPHAGE SPREADING BY IMAGE ANALYSIS DETERMINATION OF THE NUCLEO-CYTOPLASMIC RATIO

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ABSTRACT

A method is proposed for measuring the nucleo-cytoplasmic ratio automatically. the examined samples are macrophages of mice obtained from the peritoneal cavity. A computer program has been devised for the measurement of the spreading of these macrophages using a Quantimet 720 analyser. Comparison with results obtained manually allowed us to demonstrate the reliability of the automatic analysis.

INTRODUCTION

Macrophage spreading is one of the parameters measured for the study of the effect produced by drugs on macrophages (EISEN and NORONHA-DUTRA, 1977 ; FAUVE and HEVIN, 1977 ; STAHL et al, 1978). Morphological criteria have been previously described by RABINOVITCH and DE STEFANO (1973). A semi-quantitative study has been performed by STAHL et al (1980 ; 1981). The nucleo-cytoplasmic ratio could be one of the parameters used to express macrophage spreading and its measurement could complete the data previously presented. However, this ratio is difficult to measure accuretely by eye and so until now quantitative results have not been obtained. Various morpho-metrie appoaches could be applied here but an automated procedure should be developed because manual measurements may be to slow and inaccurate for the processing of large numbers of samples.

MATERIAL AND METHODS

Macrophages were obtained by rinsing the peritoneal cavity of 4 week-old female C 57 BL/6 mice with 2 ml of Stoker medium containing 2 % bovine serum albumin (BSA) (pH = 7.2). Aliquots (20 µl) of the cell suspension (2. 10^6 per ml) were placed into the microwells of microprint glass slides (Poly Labo) and incubated under individual coverslips in 5 % CO_2 atmosphere at 37° C for 30 minutes.

After rinsing in saline, adherent cells were fixed by dipping the slides for 30 minutes into PBS containing 2. 5. % glutaraldehyde at room temperature. The staining of cells is crucial since automatic image analysis requires that the backgroud, cytoplasm, and nucleus appear to the television camera as distinctly different shades of grey. For staining we used a 2 % solution of Coomassie brilliant blue R 250 in 46.5 ml methanol, 46. 5 ml distilled water, and 7 ml acetic acid (PENA, 1980). The solution was filtered and stored at room temperature. After 30 minutes staining, the smears were dehydrated by washing with alcohol and xylene prior to mounting using Techicon mounting medium.

A schematic diagram of the hardware involved in the assay is provided in Figure . The measurement involved a Zeiss microscope, Plumbicon 720 line camera, Quantimet 720 image analyser (FISCHER 1971 ; 1978) (WADLOW et al., 1972), PDP 11/34 computer with a memory of 32 K words, a VT 100 video terminal and a LA 120 line printer (1).

The image produced by the microscope is swept by the camera and submitted to the Quantimet as 600,000 image points wich are analysed in real time by the Quantimet modules. The image is first cleaned up electronically by the shading corrector, to compensate for unavoidable lighting and electronic distortions. Each of the 600,000 image points is assigned an integer from 0 to 63 according to its brightness dark to light. Two thresholds of brightness are manually set (A and B) and the 2 D detector accepts for analysis all points in whichever one of the six possible brightness ranges delimited by 0, A, B, and/or 63. Since ideally the background appears light, the cytoplasm grey, and the nucleus dark, this allows one to analyse separately the entire cell without background or just the cytoplasm, or the nucleus. The Standard Analyser identifies as a single entity (e. g., a cell) sets of contiguous image points that are in the desired range of brightness, the frame generator allowing one to discount objects that are not entirely contained in the field of vision. The surface areas and perimeters of these entities are then measured by the function analyser and relayed to computer via Field/Image/Feature interface.

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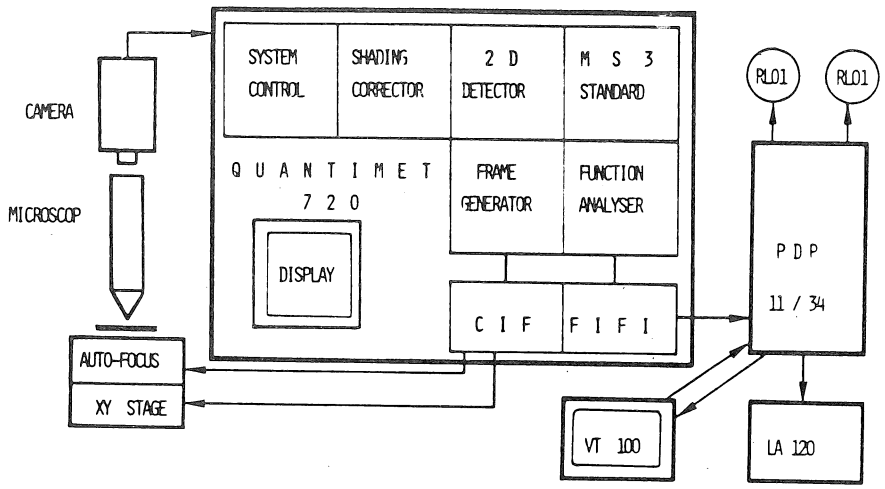
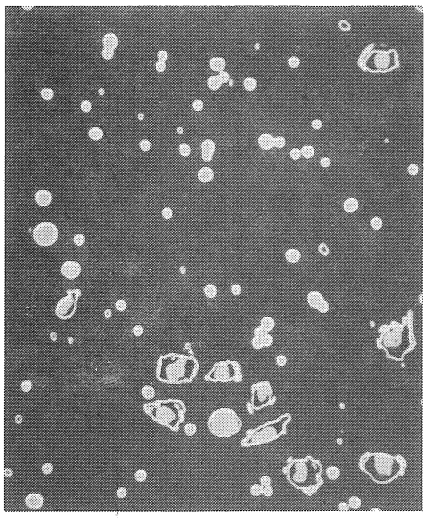


FIG 1 . System configuration

The computer coordinates all the Quantimet modules, as commanded by our computer programm MACRAP, through the Control interface module . The microscope stage is moved automatically by XY Stage MK II, and the autofocus ensures that the sharpest image. The image points can be viewed on the display at any stage of the processing, allowing one to see the quality of the focus, the objects currently being measured, etc.

The measurement involved the manual setting of the brightness thresholds A and B for eache sample so that we could single out either entire cells (darker than B) or just the nuclei (darker than A). This was easily and quickly donne by eye provided that the staining of cells was good (Fig 2).

a)



b)

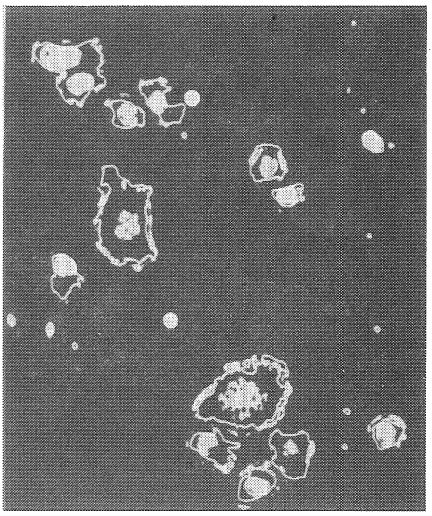


FIG 2 . Display screen of the macrophages
Spreading is determined using a 2 % BSA culture medium.

a) Normal macrophages b) Spread macrophages

When the perimeter (P) and the surface area (S) of each cell and each nucleus were mesured, a coefficient of roundness was calculated for each cell $p^2 / 4\pi S$, and the computer then decided which objects shoulds be considered as single macrophages according to preset morphological criteria. Lymphocytes are eliminated from the analysis taking account of morphological and size criteria. For the cells which were identifield as macrophages, the nucleo-cytoplasmic ratio was calculated and the microscope stage was moved to the next position. Each microwell was divided into 100 fields containing about 5 to 20 macrophages each. After completin the enumeration of a single microwell, which took about 2 minutes, the computer printed out statistical information and a histogramm of the nucleo-cytoplasmic ratio of all the identified macrophages. The significance of the results was assessed with a χ^2 test.

RESULTS

We have improved the quality of identification of the different cell types. This test was performed on 2, 336 different cells. The determinations of the biologist and of the MACRAP programme were compared for these values. the results are presented in a confusion matrix (Table 1). Column headings express the biologist's interpretation and horizontal line headings the programm responses. The erythrocytes are easily recognized whereas some little particles can be confused. The percentage of lymphocytes confused with erythrocytes or macrophages is very low (5.1 %). The percentage of macrophages confused with lymphocytes is some what higher (7.7 %). The density of the cells on the slides is needs to the sufficient to allow a large percentage of macrophage rejection (40. 7 %).

The number of each cell type is determined by the frequency of these cells on the preparation. As an example, the number 16 indicates that 16 cells identified as lymphocytes by the biologist have been recognized as macrophages by MACRAP program.

PROGRAM'S INTERPRETATION	BIOLOGIST'S INTERPRETATION ERYTHROCYTES	LYMPHOCYTES	MACROPHAGES	OTHERS	TOTAL	ERRORS %
ERYTHROCYTES	7	0	0	14	21	66.7
LYMPHOCYTES	1	75	1	2	79	5.1
MACROPHAGES	0	16	1081	74	1171	7.7
REJECTED	5	31	440	589	1065	44.7
TOTAL	13	122	1522	679	2336	
ERRORS %	7.7	13.1	.1	13.3		

The spreading of macrophages was obtained using a culture medium (Fig 3) with or without BSA. This allowed us to compare two different groups of preparations with more or less spread macrophages. The difference between the two histograms is highly significant ($P < 0.001$) using the χ^2 test. Spread macrophages (1, 766) and normal macrophages (1055) were examined. In order to compare the two histograms, the results were expressed in terms of percentage of cells compared to the total number. The two curves (Fig. 3) show a different nucleo-cytoplasmic ratio in the two cell populations. This is responsible initially for a rapid slope in the curve which

extends furthermore in the direction of the lowest value of nucleo-cytoplasmic ratio. The surface areas (1-2) obtained by overlapping the two curves are similar (1 = 15. 4 % ; 2 = 15. 1 %).

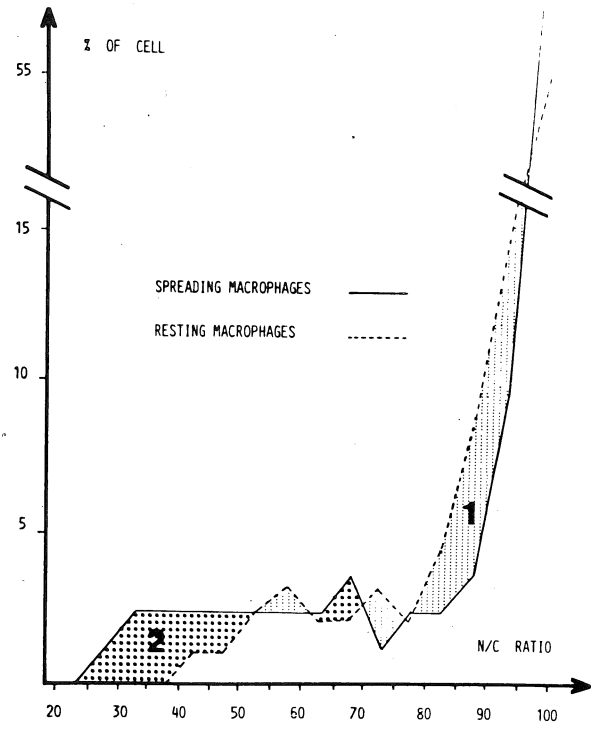


FIG 3 . Histogram of the different culture conditions according to the nucleo-cytoplasmic ratio. Comparison between the spreading and normal cell population. THe overlapping of the two curves shows that the two surface areas are similar.

DISCUSSION

The two majors problems encountered in designing the program were how to properly identify the nucleus and the cytoplasm of a cel and how to distinguish the macrophages from the lymphocytes, the erythrocytes and the impurities that accompany them. The first problem was resolved by good staining which makes black ground, the cytoplasm and the nucleus distinct from each other through different shades of grey as seen on the display screen. If that is achieved, it is simple to set the appropriate brightness threshol by eye. The staining method described above was satisfactory most of the time, but could be improved. The second problem, that of cloosing the best numerical morphological criteria for distinguishin macrophages was more difficult. To this end a program was written so

that the operator could identify cells by eye and tell the computer their type. After processing several hundred cells the computer printed out all various morphological constants that were mesured according to the cells types (surface area of nucleus, cytoplasm and cell, as well as their perimeters and their degree of roudness). From this data satisfactory criteria were estabilished for identifying macrophages, although the computer still occasionally confused contracted macrophages with lymphocytes or clusters of erythrocytes. Our results show, however, that the computer's identification agrees well with the manual identification. To maintain reliability, the program MACRAP supplied an option where the mesurement could be slowed down and the computer would identify on the screen how it had identified each object (i. e. as macrophage, lymphocyte, erythrocyte or impurity). In this manner, the operator can check the reliability of the computer's performance. We exercised this option on at least one microwell of each slide examined. We noted finally that the effect of macrophage spreading was easily observed as a displacement in the nucleo-cytoplasmic ratio histograms, and in good agreement with the manual analysis.

Under this conditions, the two histograms obtained using the MACRAP program are significantly different. The overlapping of these curves delimit two similar areas. Thos observation confirms the fact the spreading of macrophages is related to a decrease in the population with a high nucleo-cytoplasmic ratio. Altogether these results confirm the reliability of this image analysis program.

In conclusion, this automatic image analysis method for quantifying the effect of macrophage spreading allows a speed and precision not possible by manual methods. The reliability of cell identification does not suffer too much suggesting that the program can be applied for routine examination.

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