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Kinetics and Characteristics of Murine Macrophage-Lymphocyte Interaction in Presence of PHA: Appearance of Multinucleated Giant Cells *in vitro*

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The appearance of cellular associations between macrophages and lymphocytes —which we have denominated macrophage-lymphocyte rosettes— and their kinetic formation in the presence of phytohemagglutinin (PHA) have been studied in B10 A (4R) mice. The greatest number of macrophage-lymphocyte rossettes was found from 6 to 12 hours after incubation with PHA. During this time, 42.38 ± 10.70 of the total number of macrophages had lymphocytes attached to their membranes. This percentage decreased to 17.33 ± 2.07% after 24 hours. The activation of macrophages after PHA treatment was tested by the phagocytic capacity of these cells. This activity increased significantly 24 hours after incubation.

In our assay, an increase in the appearance of multinucleated giant cells when compared to controls was also observed. When the macrophages were lymphocyte depleted, the appearance of the multinucleated giant cells was significantly lower. The kinetics for these formations are also discussed.

The role of the macrophage in immune response initiation has been clearly established in the last few years (12, 16). It is known that macrophages act as target cells for different factors released by the activated lymphocytes (lymphokines) (2). It has also been demonstrated that lymphocyte response induced by mitogens such as phytohemagglutinin (PHA) is macrophage dependent (11, 18). Although the intimate mechanisms of this collaboration have not been determined, it might be mediated by soluble factors released by macrophages (7) or by a direct contact between both types of cells (13). The direct interactions between macrophages and lymphocytes have been described as spontaneous (1) or in response to different antigens (17).

Moreover, the origin and formation mechanism of multinucleated giant cells

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(MGC's) first reported by ROKITANSKY (10) and LANGHANS (4, 5) over a century ago still remains unclear. These MGC's are recognised as a common feature of granulomas induced by immunological and non-immunological stimuli (15). It has been observed that crude supernatants from cultures of antigen-stimulated lymph node cells of rabbits induce alveolar macraophages to form giant cells *in vitro* (3, 8). Recently, POSTLETHWAITE *et al.*, (9) reported the formation of MGC's from human monocyte precursors mediated by a protein released from antigen or mitogen stimulated lymphocytes.

This paper presents an assay which allows for the study of celullar associations between peritoneal macrophages and lymphocytes in B10 A (4R) mice in response to a mitogen as PHA; the rate of MGC formation was also observed.

Materials and methods

The cells were obtained from B10 A (4R) mice by peritoneal washing with RPMI 1640 medium (Difco) containing cloxacillin-ampicillin (10 μg/ml), Hepes buffer and 10% fetal calf serum (Difco) (heat inactivated at 56° C for 30 min). The cellular suspension was adjusted to 0.5 × 10^6 macrophages/ml. Aliquots of 200 μl were then placed in MIF plates (Sterilin); PHA (at a final concentration of 0.07 μg/ml) was added after 45 minutes of preincubation at 37° C in a humidified atmosphere containing 95% aire and 5% CO_2. Identical amounts of phosphate buffer saline solution (PBS) were dispensed in the control wells.

Cellular Associations and Multinucleated Giant Cells. The plates were washed in PBS at 37° C after 2, 6, 12 or 24 hours of incubation and the adherent monolayer fixed and stained with Giemsa (Dade-Grifols S.A.). The number of macrophage-lymphocyte rosettes (M-L rosettes) —macrophages with one or more lymphocytes attached to their membranes (fig. 1a)— was determined in an optical microscope (Olympus, Japan).

The rosettes were quantified as percentages with respect to macrophage totality. A minimum of 300 macrophages were counted.

Some cultures were developed in suspended drops at the times indicated above and then smeared, fixed and stained for a better observation of the association between the macrophages and lymphocytes (fig. 1a).

The number of multinucleated giant cells (MGC's) —cells containing two or more nuclei— was scored from the total number of macrophages counted (fig. 1b). Cultures were also lymphocyte depleted by several washings in the medium after 45 minutes of preincubation.

Phagocytosis. The cellular suspension obtained as indicated above from the peritoneum after 24 hours of incubation was washed in PBS at 37° C to eliminate nonviable or non —adherent cells. 200 μl medium and 20 μl latex particles (with a diameter of 0.8 μ and 1% PBS) were dispensed into the wells and at the end of different phagocytosis times (5, 10, 15, 20, 25 or 30 min) the plates were washed again and stained with Giemsa. The number of particles phagocyted was counted and expressed as the number of particles per 100 macrophages.

Statistical Analysis. The statistical study was done according to Student's *t* test, $p < 0.05$ being the minimum significant level.

Results

Macrophage - Lymphocyte Rosettes. Figure 2 shows the percentage of macrophage-lymphocyte associations-denominated «rosettes» because of their simi-

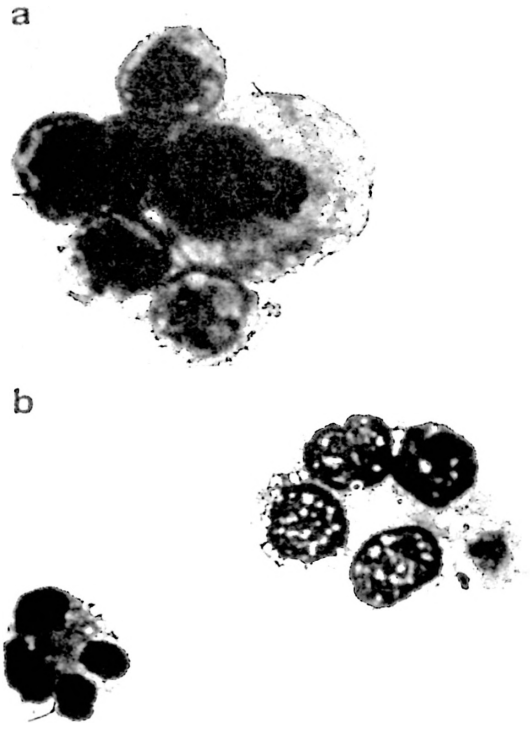

Fig. 1. *Photomicrograph of murine peritoneal cell suspension.*
(a) Macrophage-lymphocyte rosette in cultures with PHA developed in suspended drop. (b) A multinucleated giant cell.

larity to other cellular associations between different cellular types. The greatest number of rosettes (42.38 ± 10.70) was observed 6 hours after incubation. This percentage decreased to 17.33 ± 2.07% after 24 hours. There are statistically significant differences ($p < 0.001$) with respect to controls in all incubation times studied.

Multinucleated Giant Cells. The percentage of MGC's at different culture times is shown in figure 3. There are statistically significant difference ($p < 0.001$) after 24 hours of incubation with PHA as the number of these types of cells is higher (15.95 ± 1.40%) when compared to controls (5.36 ± 1.30%).

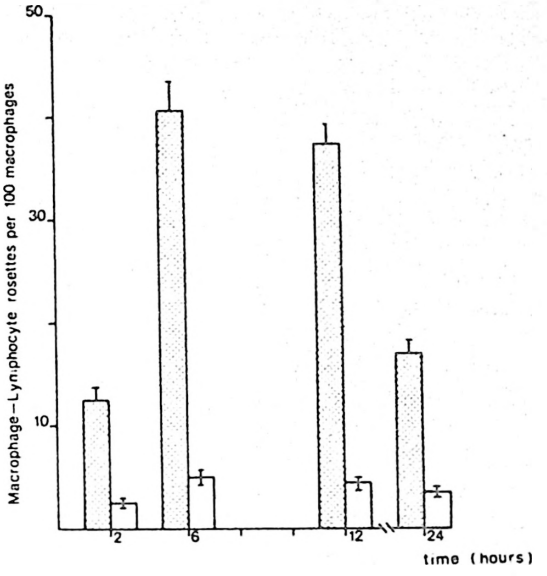

Fig. 2. *Kinetics for cellular macrophage-lymphocyte associations in cultures with PHA (dotted column) or PBS (blanck column).*
Each value is the mean ± S. D. of 5 experiments.

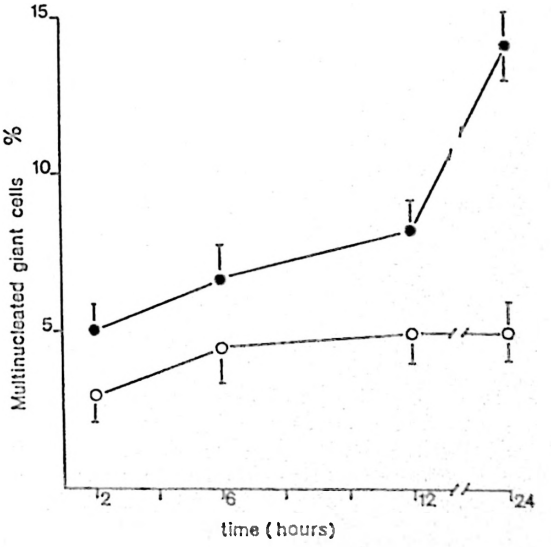

Fig. 3. *Kinetics for multinucleated giant cell appearance in culture with PHA (●) or PBS (○).*
Each value is the mean ± S. D. of 5 experiments.

Table I. *Number of multinucleated giant cells per 100 macrophages cultured in the presence or absence of lymphocytes.*
The values are the mean ± S.D. of 5 experiments.

Cell suspension	Multinucleated Giant Cells (%) Controls	Incubated with PHA
Macrophages and lymphocytes	5.36 ± 1.3	15.95 ± 1.40
Macrophages alone	4.52 ± 2.1	5.80 ± 1.60

Moreover, in controls the number of nuclei found in these giant cells was generally 2 while in PHA stimulated cultures this number oscillated between 2 and 10, these really being considered authentic giant cells.

The percentage of MGC's after 24 hours of culture in lymphocyte depleted peritoneal cell suspension is similar to the control plates (without PHA) and statistically lower ($p < 0.001$) than the macrophage-lymphocyte cultures with PHA (Table I).

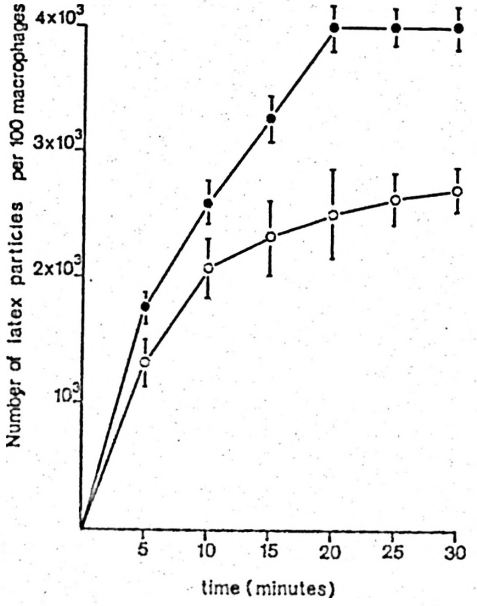

Fig. 4. *Phagocytic capacity of macrophages after 24 hours in culture with PHA (●) of PBS (○).*
Each value is the mean ± S. D. of 5 experiments.

Phagocytosis. Macrophages showed a higher phagocytic capacity with respect to controls after 24 hours of incubation with lymphocytes and PHA (Figure 4). This difference is statistically significant ($p < 0.05$) after 10 minutes of incubation, being highly significant ($p < 0.001$) after 20 minutes.

Discussion

We have developed an assay whereby the number of M-L rosettes and multinucleated giant cells produced by a known number of macrophages can be measured. The assay involves cultures of macrophages and lymphocytes incubated at different times with PHA. The results obtained with this method concurs with previous studies by other authors who also found cellular associations between macrophages and lymphocytes in the presence of antigens (6). The associations might represent the *helper* role of the macrophage and thus support the hypothesis that direct contact between macrophages and lymphocytes is necessary to achieve an effective lymphoproliferative response (13).

Considering that lymphocytes are early activated (14), they might have become activated after 24 hours in PHA, thus rendering direct contact with macrophages unnecessary. This would explain the kinetics for the appearance of rosettes found in our experiments with maximum percentage from 6 to 12 hours of incubation (Fig. 2). It should be noted that this decrease was not due to a disminished macrophage activity as these cells had a high phagocytic capacity at this time (Figure 4).

Our assay also allows for the study of the kinetics for the appearance of multinucleated giant cells *in vivo*. Although the percentage of MGC's was higher in the cultures with PHA, the difference was not statistically significant until after 24 hours of incubation. This may be ex-

plained by the release into the culture medium of a lymphokyne responsible for the transformation from a macrophage to a MGC, and not to a direct effect of PHA on the macrophage. The fact that lymphocyte depleted peritoneal macrophages incubated with PHA for 24 hours did not form any MGC's in the culture also supports this hypothesis.

The production of a factor by antigen stimulated peripheral blood lymphocytes promoting the formation of MGC's from human monocyte precursors has recently been described (9). Thus a similar factor could be responsible for the formation of MGC's in our assay with murine peritoneal cells. These studies could provide a basis for postulating that macrophages may accumulate in granulomas in response to chemotatic factors derived from the lymphocytes retained by MIF, to then fuse themselves and form MGC's.

Our study demonstrated that M-L rosettes and MGC's *in vitro* are formed from murine peritoneal macrophages in the presence of lymphocytes and PHA. These results may facilitate future studies about the interaction between macrophages and lymphocytes as well as the biological features and functions of MGC's.

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Resumen

Se estudia en ratones B10 A (4R) la aparición de asociaciones celulares entre macrófagos y linfocitos que denominamos «roseta macrófago-linfocítica», así como su cinética de formación en presencia de fitohemaglutinina (PHA). El mayor número de estas agrupaciones se encuentra entre las 6 y 12 horas de incubación con PHA en las que el 42,38 ± 10,70% del número total de macrófagos tienen linfocitos unidos a sus membranas. A las 24 horas este porcentaje desciende a un 17,33 ± 2,07%. La activación de los macrófagos tras el tratamiento con PHA se estudia mediante su capacidad fagocítica que se encuentra significativamente elevada a las 24 horas de cultivo.

Por otra parte, se observa también un incremento en la aparición de células gigantes multinucleadas en este sistema. La depleción de linfocitos produce un descenso significativo de estas células gigantes multinucleadas. Se discute su cinética de aparición.

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