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Microtechnique for Studies on the Role of Monocytes in the Stimulation of Lymphocytes

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A microtiter technique was used to measure lymphocyte stimulation by antigen-pulsed monocytes. Monocytes from human blood were isolated on polyester film disks. The disks were exposed to antigen and incubated together with lymphocytes in the wells of a microtiter plate. The method was technically simple and required no expensive materials.

The presence of monocytes has been found to be necessary in order to get an antigen to stimulate lymphocytes (3, 6, 13). The antigen first binds to the monocytes or macrophages, and then these cells induce immunologically committed lymphocytes to proliferate (8, 12, 13). The mechanism behind the cellular interactions is still unclear (12).

In studies on these interactions in vitro, monocytes are first treated with antigen and then incubated together with lymphocytes. Cells from exudates or from blood of various mammals are used (9). From human blood a mixture of monocytes and lymphocytes is first prepared by isopycnic centrifugation (2), and then monocytes are separated from this mixture by using their ability to adhere to various surfaces (1). In one well-established in vitro model, monocytes are isolated on a glass slide, and the slide is treated with antigen. Then it is inserted in a flat-bottomed Leighton tube (1), in which lymphocyte stimulation is induced (4).

This technique is elaborate, and experiments cannot be run in large series. Lymphocyte stimulation can successfully be studied by microtiter techniques (5). The present report describes a technique for studying monocyte-lymphocyte interactions in microtiter plates. Monocytes are allowed to adhere to polyester film disks which can then be treated with antigen and incubated with lymphocytes in the wells of a microtiter plate.

MATERIALS AND METHODS

Hanks balanced salt solution and RPMI 1640-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (RPMI-HEPES) were purchased from Grand Island Biological Co., Madison, Wisc. Ficoll-Isopaque was from Nyegaard & Co. AB, Stockholm, Sweden, and fetal bovine serum was from Flow Laboratories, Irwine Ayreshire, Scotland. Purified protein derivative (PPD)-tuberculin was from Statens Seruminstitut, Copenhagen, Denmark. Disks (diameter, 5.5 mm) were punched out by an ordinary perforator (catalog no. 9386; Ossvald Johnsson, Eskilstuna, Sweden) from a polyester film (projection film for plain paper copier 400; catalog no. 6290; 0.10 mm; Rosendahls Fabriker AB, Filipstad, Sweden). An antistatic pistol (Zerostat) was from Zerostat Instruments Ltd., Huntingdon, Camts, England. Microtest II tissue culture plates and films for closing the plates were from Falcon Plastics, Los Angeles, Calif. Carbonyl iron powder, grade SF, was from GAF Corp., New York, N.Y., and a magnet (catalog no. 939) was from James Neill Sheffield Ltd., Sheffield, England. Silicone compound no. 7 was from Dow Corning Ltd., Barry, Glamorgan, United Kingdom, and a synthetic mounting medium (Pertex) was from Histolab, Gothenburg, Sweden.

Culture medium. The culture medium consisted of 85% RPMI-HEPES and 15% pooled inactivated (56°C, 30 min) human serum. There was 100 μg of streptomycin sulfate per ml of medium.

Preparation of mononuclear leukocytes. Samples (100 ml) of venous blood from healthy blood donors were defibrinated by gentle agitation with glass beads. Mononuclear leukocytes were separated by centrifugation on Ficoll-Isopaque (2), washed twice in Hanks balanced salt solution, and suspended in culture medium. The density of mononuclear leukocytes was 5×10^6 cells per ml of medium.

Adhesion of monocytes to disks. Monocytes were separated from the mononuclear leukocyte suspension by using their adhering ability (11). Plastic disks were heat disinfected (70°C, 1 h), and one disk was placed at the bottom of each well of a tissue culture plate. Static charge on the disks was neutralized by an antistatic pistol. A 100-μl amount of the suspension was added to each well, and wells were closed with film and incubated overnight at 37°C. To remove nonadhering cells, the disks were gently rinsed in RPMI-HEPES at room temperature. Some of the disks were glued to a slide by Pertex and stained by Giemsa stain or for nonspecific esterase (10).

Antigen treatment of adhered monocytes. Immediately after rinsing, each disk was added to a petri dish which contained a solution of PPD in RPMI-HEPES at 24 or 37°C. Treatment at 0°C was performed in a solution of PPD in glass tubes kept in ice.

After incubation for various times, each disk was gently rinsed in RPMI-HEPES and placed in a well at the bottom of which a droplet of silicone grease had been added and which also contained 100 μl of culture medium. Care was taken to avoid drying of the disk.

Preparation of lymphocytes. Lymphocytes were prepared from a suspension of mononuclear leukocytes. To remove phagocytes, the mononuclear leukocytes were incubated with 0.02 g of iron particles per ml of suspension (7). After incubation for 30 min at 37°C with gentle agitation at intervals of 5 min, phagocytic cells were removed by a magnet.

The lymphocytes were washed twice in RPMI-HEPES, suspended in culture medium, and stored overnight at room temperature.

Lymphocyte cultures. A 100-μl amount of lymphocyte suspension was added to each well containing a disk with PPD-pulsed or nonpulsed monocytes in 100 μl of culture medium. Some wells contained a disk with nonpulsed monocytes and 200 μl of a solution of 50 μg of PPD per ml of culture medium. This dose of PPD had been found to induce a lymphocyte stimulation which was about optimal. The final density of lymphocytes was 1.5×10^6 cells per ml of medium. The tissue culture plates were closed with film and incubated at 37°C for 6 days. To estimate DNA synthesis of the lymphocytes, 0.04 μCi of [^{14}C]thymidine in 20 μl of RPMI-HEPES was added to each culture 6 h before end of the incubation period.

Cultures were washed and collected onto glass fiber filters by a semiautomatic multiple-sample processor (5). The filters were placed in 10 ml of scintillation fluid and counted in a scintillation counter. Quenching, estimated by the external standard method, was very similar throughout the study, and therefore results were expressed as counts per minute. Three to five replicated cultures were assayed for deoxyribonucleic acid (DNA) synthesis.

RESULTS

Nonspecific esterases were present in more than 90% of cells that adhered to the disks. These cells could be considered to be monocytes. When such a disk was treated with PPD, rinsed, and added to a culture of lymphocytes, it induced the incorporation of [^{14}C]thymidine into the DNA of the lymphocytes (Table 1). The incorporation was much higher than that induced by a nonpulsed disk, and it was about as high as that induced by soluble PPD in the presence of 5×10^5 mononuclear leukocytes as the source of monocytes. When the disks were prepared in wells containing various numbers of mononuclear leukocytes and then treated with PPD, disks prepared in the presence of 10^5 to 10^6 mononuclear leukocytes induced the highest incorporation of thymidine into lymphocyte DNA (Fig. 1).

The lymphocyte response also depended on the concentration of PPD used in the treatment of monocytes (Fig. 2). It increased with concentration in the range of 0.4 to 50 μg of PPD per ml of medium, was optimal at 50 μg/ml, and was suboptimal at 250 μg/ml.

The effects of temperature and length of PPD treatment of monocytes on the incorporation of [^{14}C]thymidine were studied. Incorporation was higher after treatment at 37°C than after treatment at 24°C, whereas treatment at 0°C for up to 4 h resulted in an incorporation which was only slightly higher than that induced by nontreated disks (Fig. 3A). The incorporation increased with length of treatment. At 37°C 1 h of treatment was enough to yield an optimal incorporation, whereas at 24°C a higher incorporation was obtained after treatment for 4 h than after treatment for 1 h (Fig. 3B).

TABLE 1. *Stimulation of lymphocytes by a polyester film disk with PPD-pulsed monocytes*[a]

Expt no.	[^{14}C]thymidine incorporation (cpm, $\times 10^{-3}$) in the following cultures:		
	Lymphocytes + plastic disk with PPD-pulsed monocytes	Lymphocytes + plastic disk with nonpulsed monocytes	Lymphocytes + plastic disk with nonpulsed monocytes + 50 μg of PPD per ml of culture
1	2,242 ± 89[b]	122 ± 26	2,443 ± 432
2	1,861 ± 165	138 ± 27	2,014 ± 245
3	4,395 ± 613	441 ± 47	2,809 ± 741

[a] Pulsing was performed for 5 h at 37°C. Cultures were incubated for 6 days at 37°C, and then the incorporation of [^{14}C]thymidine was estimated.
[b] Mean ± standard error of four cultures.

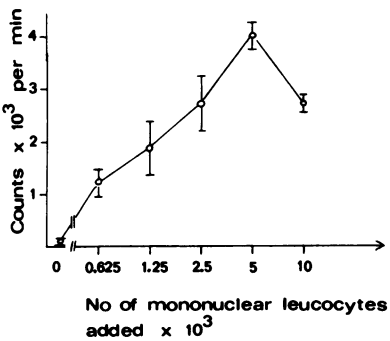

FIG. 1. *Stimulation of lymphocytes by various numbers of PPD-pulsed monocytes. From 0 to 10^6 mononuclear leukocytes were added to each well, and 32% of the cells were monocytes; 10 to 20% of the monocytes adhered to the disks. The incorporation of [^{14}C]thymidine into the DNA of the lymphocytes was measured, and counts per minute ($\times 10^{-3}$, ± standard error of the mean of three cultures) were plotted against the number of mononuclear leukocytes added per well.*

DISCUSSION

The lymphocyte response to antigen-pulsed monocytes was easily assayed by the microtiter technique. Monocytes were isolated on polyester film disks, treated with antigen, and added, together with lymphocytes, to the wells of a microtiter plate.

When the monocytes were exposed to PPD at 37°C, 1 h was enough to induce an optimal lymphocyte stimulation. Exposure at 24°C was less efficient, and almost no stimulation was obtained after exposure at 0°C. These results are in accordance with results obtained by a macrotechnique in which Leighton tubes are used (4). Variations in the number of monocytes and the dose of PPD also gave results similar to those obtained with the macrotechnique.

PPD, which is a soluble agent, was used as an antigen in all of the present experiments. The present procedure has, however, been used in our laboratory also in studies on the interactions of membrane antigen from *Francisella tularensis*, monocytes, and sensitized T lymphocytes (14).

Lymphocyte stimulation is widely assayed by the microtiter technique. The present procedure requires only a cheap and easily produced supplement to this technique. The plastic material is commercially available as a projection film, and the disks are prepared by using an ordinary paper perforator.

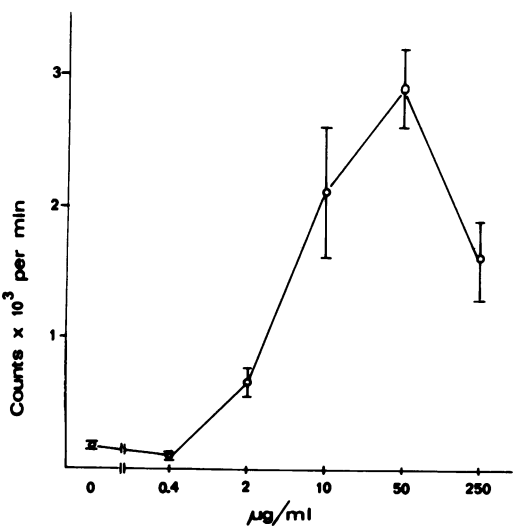

FIG. 2. *Stimulation of lymphocytes by plastic disks with monocytes pulsed with various concentrations of PPD. The incorporation of [^{14}C]thymidine into DNA of the lymphocytes was measured, and counts per minute (×10^{-3}, ± standard error of the mean of five cultures) were plotted against the concentration of PPD.*

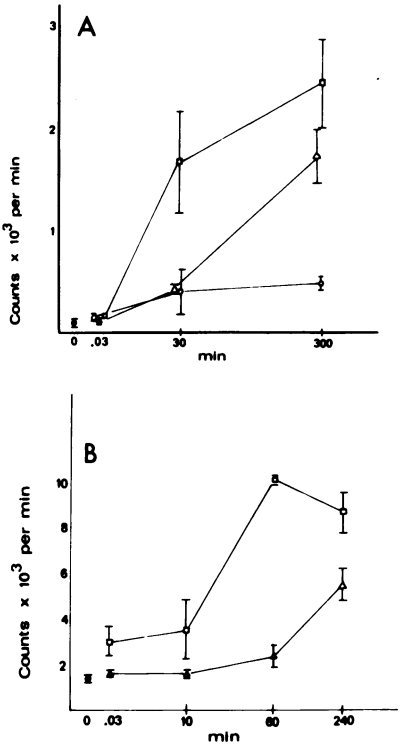

FIG. 3. *Stimulation of lymphocytes by plastic disks with monocytes pulsed with PPD for various times at 0 (○), 22 (△), and 37°C (□). The incorporation of [^{14}C]thymidine into the DNA of the lymphocytes was measured, and counts per minute (×10^{-3}, ± standard error of the mean of five cultures) are indicated. The two experiments (A and B) were done using blood cells from different individuals.*

The present procedure may become useful in studies of the interactions among antigen, monocytes, and various populations of lymphocytes. Furthermore, it may be used in experiments in which the aim is to find the target cell of immunoregulatory agents. In such experiments monocytes or lymphocytes or a mixture of these cells may be exposed to the test agent during various intervals of the stimulation process. For this purpose a microtechnique seems suitable, since the investigation of dose-response relations and kinetics may require a large number of cultures within each experiment.

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