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Activation of human peripheral blood lymphocytes by concanavalin A dependence of monocytes

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SUMMARY

The activation of human peripheral blood lymphocytes or isolated T lymphocytes by concanavalin A (Con A) is highly potentiated by the presence of autologous, mitomycin C-treated monocytes. The optimal lymphocyte:monocyte ratio within a broad dose range is 1:1 when the incorporation of [^{14}C]thymidine is expressed as total incorporation per culture tube and 1:10 when expressed per lymphocyte. A five- to ten-fold increase of total DNA synthesis is noted in the presence of 10–90% monocytes. The data may help to explain the wide variations in Con A responsiveness of human peripheral lymphocytes which may be partly related to differences in purification which give rise to cell preparations containing varying amounts of monocytes.

INTRODUCTION

Mitogens of plant or bacterial origin are known to stimulate lymphocytes to blast transformation and DNA synthesis *in vitro*. The response may vary in different species and also in cells from different sources in the same animal (for review, see Möller, 1972). Soluble concanavalin A (Con A) selectively stimulates thymus-derived lymphocytes (T cells) in mice (Stobo, 1972). The mitogen-induced T-cell response has been considered independent of adherent cells contrasting to the antigen-induced activation of lymphocytes from immunized animals (Rosenthal & Shevach, 1973). However, in a recent study it was shown that removal of non-T cells from human peripheral blood lymphocytes markedly reduced the thymidine incorporation after activation with Con A (Hedfors, 1974). The addition of autologous, adherent leucocytes to purified T lymphocytes highly potentiated the response (Hedfors, 1974).

In the present study the potentiation of Con A-induced DNA synthesis in human peripheral blood lymphocytes by blood monocytes has been investigated further.

MATERIALS AND METHODS

Preparation by lymphocytes. Lymphocytes were isolated from defibrinated venous blood by sedimentation through gelatine, followed by iron treatment to remove phagocytic cells. The method has been described in

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detail earlier (Holm *et al.*, 1975). By morphological criteria and functional tests comprising phagocytosis of *Candida albicans*, these preparations contained >99% small lymphocytes.

Fractionation of lymphocytes on immunoglobulin–anti-immunoglobulin-coated columns. Acid-treated glass beads (Superbrite, 3M Company, Minneapolis, Minnesota) were incubated with 0·5% human IgG in phosphate-buffered saline, pH 7·4 (PBS) for 1 hr at 45°C followed by overnight incubation at 4°C. After washing with PBS, 50 ml of beads were poured into a column (15 × 30 cm) and treated with low dilutions of hyperimmune rabbit anti-IgG serum for 1 hr. The excess of antiserum was washed off and the column filled with PBS. 100×10^6 lymphocytes were applied to the column and eluted with Hanks's solution with 0·15 M Tris buffer, pH 7·4 (HT) and the flow rate of 2–3 ml/min. For further details of the method, see Wigzell, Sundqvist & Yoshida (1972). Aliquots of the unfractionated and immunoglobulin–anti-immunoglobulin column-fractionated lymphocyte preparations were treated with 0·83% ammonium chloride for 5–10 min to lyse remaining red blood cells (Boyle, 1968). The preparations were then examined for the presence of lymphocytes having receptors for activated human complement (C3).

Complement receptor binding lymphocytes. Sheep red blood cells (SRBC) coated with rabbit anti-SRBC serum were incubated with fresh human AB serum as a source of complement to form EAC cells. 0·25 ml EAC was mixed with 0·25 ml (10^6) lymphocytes, pelleted down and incubated at 37°C for 15 min. After vigorous resuspension, 200 cells were counted on a glass slide. Cells binding more than three EAC were regarded as positive (EAC-binding lymphocytes) (Holm *et al.*, 1975). Fractionated lymphocyte preparations containing >3% EAC-binding lymphocytes were excluded. The trypan Blue exclusion test revealed >98% viable cells.

Isolation of monocytes. Heparinized venous blood was sedimented through gelatine (Coulson & Chalmers, 1964). The leucocytes were washed once in Tris-buffered Hanks's solution, pH 7·4 (HT) with heparin, 50 i.u./ml. The cells were layered on a bovine serum albumin gradient and centrifuged at 1·700 *g* for 40 min at room temperature. The top layer containing monocytes and lymphocytes was washed twice in HT with 1% gelatine, suspended in HT with 50% foetal bovine serum and incubated in plastic tissue culture flasks (Falcon Plastics, Los Angeles, California) for 60 min at 37°C. Non-adherent cells were removed by gentle shaking. Remaining cells were detached by vigorous shaking.

By morphological and functional criteria, the preparations contained about 95% monocytes and were contaminated mainly by small lymphocytes. For further details, see Holm (1972); Holm & Hammarström (1973). The monocytes were treated with mitomycin C (25 μg/ml medium for 30 min at 37°C) and used in combination with autologous lymphocytes at varying ratios and varying total numbers of cells per culture tube (for details, see Results section).

Culture conditions. RPMI 1640 medium with 20 mM Hepes buffer supplemented with 2 mM glutamine, 100 i.u. of penicillin, 100 μg of streptomycin per millilitre and 15% heat-inactivated human AB serum was used. The cell preparations were incubated in conical glass tubes (15 × 110 mm) with or without Con A (batch number 4000, Pharmacia, Uppsala, Sweden) at 37°C in a humid atmosphere with 5% CO_2 for 3 days. The final volume per tube was 1·5 ml. 0·1 μCi of [^{14}C]thymidine (specific activity 10 μCi/mM; Radiochemical Centre, Amersham, Bucks) was added during the last 24 hr. In addition, kinetic studies of thymidine incorporation in the various cell preparations were performed. The cultures were pulsed with 0·1 μCi [^{14}C]-thymidine (specific activity 60 μCi/mM) during 4-hr periods. DNA was extracted by a trichloroacetic acid procedure and its radioactivity measured in a Packard liquid scintillation counter. The means from duplicate incubations are presented.

Lymphocyte survival. Cultures of lymphocytes with or without monocytes were set up as described above. Before harvest a ten-fold excess of chicken red blood cells (ChRBC) was added to the tubes. The Trypan Blue exclusion test was used for estimating the viability of the cells. Thereafter smears were made on glass slides and differential count performed after staining (Nordling, Andersson & Häyry, 1972). In some experiments 20×10^6 *Candida albicans* was added per million mononuclear cells during the last 30 min of incubation. This was done to make the identification of monocytes easier.

RESULTS

The optimal stimulating dose of Con A for 5×10^5 purified unfractionated lymphocytes per tube was 20–40 μg/ml of Con A.

In a series of experiments exemplified in Fig. 1 the number of cells per culture tube was

kept constant (5×10^5) and the ratio between T lymphocytes and monocytes varied. The cultures were stimulated with 0·2–20 μg/ml of Con A. When the total thymidine incorporation was recorded, optimal DNA synthesis was noted at the lymphocyte–monocyte ratio 1:1 (Fig. 1a). However, when the DNA synthesis was expressed as counts per minute (ct/min) per lymphocyte, the optimal response was obtained at a ten-fold excess of monocytes (Fig. 1b). Twenty micrograms per millilitre of Con A was slightly superior to 2 μg/ml. In the following experiments the higher dose was used.

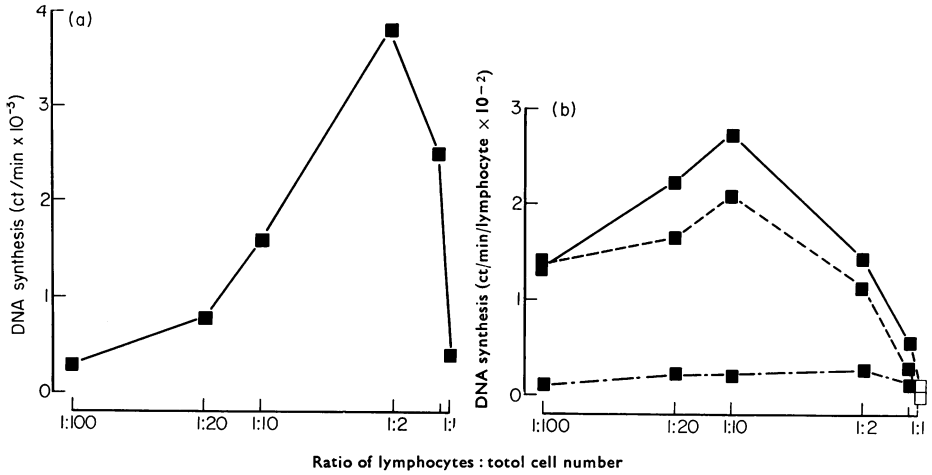


FIG. 1. (a) Total [^{14}C]thymidine uptake in ct/min $\times 10^{-3}$ of immunoglobulin–anti-immunoglobulin column-fractionated lymphocytes (□, T lymphocytes containing less than 3% non-T cells) supplemented with mitomycin-C treated, autologous monocytes (■) in response to 20 μg/ml of Con A after 3 days of culture. Five $\times 10^5$ cells were cultured per tube and the ratio between lymphocytes and monocytes varied. The figures are mean values of duplicate tubes. (b) [^{14}C]thymidine uptake is expressed as ct/min/lymphocyte $\times 10^{-2}$. (—) 20 μg; (– – –) 2 μg; (- · - ·) 0·2 μg.

In the next series of experiments a constant number of monocytes (5×10^5) was added to an increasing number of T lymphocytes or unfractionated lymphocytes. The cells were stimulated with 20 μg/ml of Con A. As shown in the experiment of Fig. 2a, the peak response expressed as ct/min per tube was obtained at the lymphocyte–monocyte ratio 1:1 and at a cell density of one million cells per tube. The total DNA synthesis in unfractionated lymphocytes was potentiated by monocytes when incubated at low cell density but not at high cell concentration. In contrast, enriched T lymphocytes which were poorly activated at all cell concentrations responded almost as well as unseparated lymphocytes in the presence of monocytes (Fig. 2a).

When incorporation of [^{14}C]thymidine was expressed as ct/min per lymphocyte (Fig. 2b), the poor stimulation of unsupplemented T lymphocytes is further illustrated. Moreover, the peak DNA synthesis was noted at lymphocyte–monocyte ratio 1:10 as in Fig. 1b.

The influence of cell density was further studied at the optimal lymphocyte–monocyte ratio 1:10. Fig. 3 shows that thymidine incorporation per lymphocyte remains constant within a ten-fold increase of cells up to $1{\cdot}5 \times 10^6$ cells per culture tube. Higher cell concentrations were inhibitory.

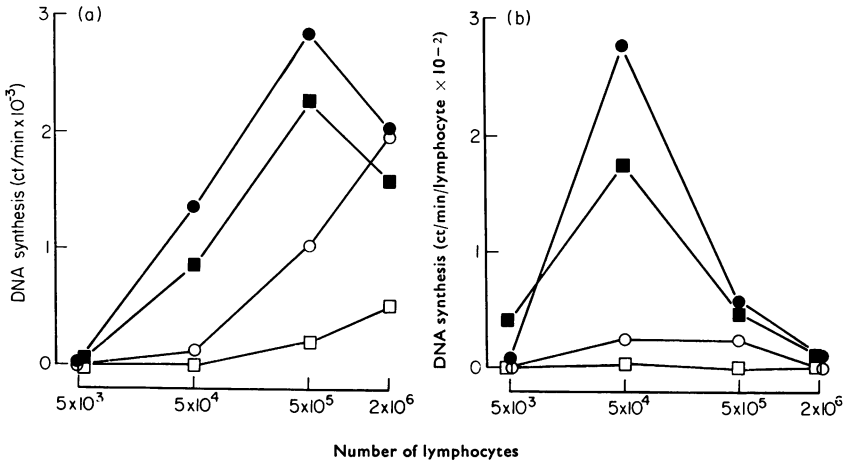
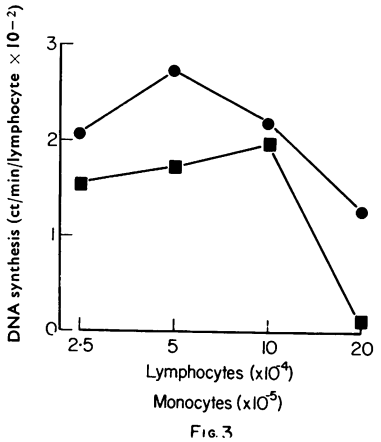


FIG. 2. (a) The total [^{14}C]thymidine uptake in ct/min $\times 10^{-3}$ of increasing number of unfractionated lymphocytes supplemented with 5×10^5 mitomycin-C treated autologous monocytes (●), T lymphocytes supplemented with 5×10^5 mitomycin-treated autologous monocytes (■), unfractionated (○) and T lymphocytes (□) in response to 20 μg/ml of Con A for 3 days of culture. The figures are mean values of duplicate tubes. (b) [^{14}C]thymidine uptake is expressed as ct/min/lymphocyte $\times 10^{-2}$ during the same culture conditions as in (a).



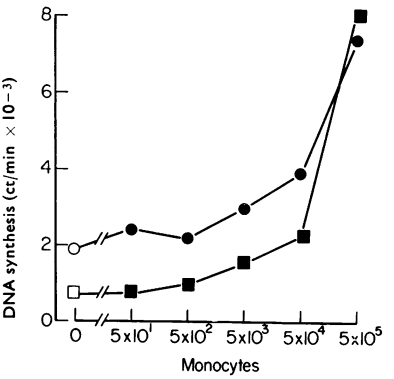


FIG. 3. [^{14}C]thymidine uptake in ct/min/lymphocyte $\times 10^{-2}$ of unfractionated lymphocytes supplemented with mitomycin C-treated autologous monocytes (●) and T lymphocytes supplemented with mitomycin C-treated autologous monocytes (■) in response to 20 μg/ml of Con A for 3 days of culture. The ratio between lymphocytes and monocytes was kept constant 1:10 and the total number of cells per culture tube increased. The figures are mean values of duplicate tubes.

FIG. 4. Total [^{14}C]thymidine uptake in ct/min $\times 10^{-3}$ of 5×10^5 unfractionated (●) and T lymphocytes (■) respectively supplemented with an increasing number of mitomycin C-treated autologous monocytes in response to Con A 20 μg/ml for 3 days of culture. The figures are mean values of duplicate tubes.

The incorporation of [^{14}C]thymidine in a constant number of T lymphocytes or unfractionated lymphocytes was only slightly improved by the addition of small numbers (<10%) of monocytes (Fig. 4), whereas a five- to ten-fold increase of total DNA synthesis was noted in the presence of 80–90% monocytes.

The early kinetics of thymidine incorporation into various lymphocyte preparations is shown in Fig. 5. DNA synthesis is measurable after 30 hr of culture in monocyte-supplemented cultures in contrast to the thymidine incorporation of isolated T cells in which significant thymidine incorporation is not measurable until 52 hr of culture (Fig. 5).

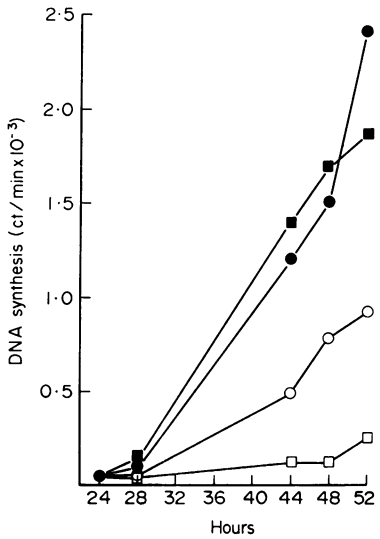


FIG. 5. Kinetics of [^{14}C]thymidine uptake in ct/min $\times 10^{-3}$ of $2{\cdot}5 \times 10^5$ unfractionated lymphocytes (□) supplemented with $2{\cdot}5 \times 10^5$ mitomycin C-treated, autologous monocytes (●), $2{\cdot}5 \times 10^5$ T lymphocytes supplemented with $2{\cdot}5 \times 10^5$ mitomycin C-treated monocytes (■), unfractionated (○) and T lymphocytes (□) in response to 20 μg/ml of Con A. In the non-monocyte-supplemented cultures the cell number was kept constant by the addition of mitomycin C-treated unfractionated or T lymphocytes respectively. The cultures were pulsed with [^{14}C]thymidine (specific activity 60 mCi/mM) during 4-hr periods.

The viability of lymphocytes after 3 days of culture amounted to 94–97% in cultures supplemented with monocytes as well as in non-supplemented cultures. Nor were any differences in cell survival encountered; the ten-fold excess of ChRBC was preserved in all combinations used.

DISCUSSION

The present experiments indicate that the stimulation of human peripheral blood lymphocytes by Con A is highly dependent on the presence of monocytes. The optimal lymphocyte–monocyte ratio within a broad range of Con A concentrations was 1:10 when thymidine incorporation was expressed as ct/min per lymphocyte. The optimal activation was dependent on the dose of Con A. However, the Con A doses giving optimal DNA synthesis were independent of cell density up to $1{\cdot}5 \times 10^6$ cells per culture tube.

The mechanism by which monocytes potentiate the DNA synthesis is not known. Reduced survival of lymphocytes in monocyte-depleted cultures seem to be a mechanism which can be excluded. Con A bound to the monocytes may present a more potent stimulus to the lymphocytes than soluble Con A (Andersson, Sjöberg & Möller, 1972). This may explain the highly potentiating effect of adherent cells when lymphocytes are stimulated with low doses of Con A (Hedfors, 1974; Lohrmann, Novikovs & Graw, 1974). The presence of monocytes may lead to recruitment of lymphocytes. Thus, the consistent observation that the DNA synthesis of unfractionated lymphocytes supplemented with monocytes was always higher than that of supplemented T lymphocytes (Fig. 2) may depend on recruitment of non-T lymphocytes.

The presence of monocytes may shorten the cell cycle of Con A-activated lymphocytes. However, the increment of DNA synthesis in monocyte-supplemented and non-supplemented cultures during the first 48 hr of stimulation was approximately the same (Fig. 5) which rather supports the same cell cycle length (Lohrmann, Graw & Graw, 1974). The longer lag phase for non-supplemented cultures before DNA synthesis was detectable may be due to different numbers of responding lymphocytes.

Finally, it cannot be excluded that the fractionation of lymphocytes through an immunoglobulin–anti-immunoglobulin column with a yield of 50% T cells removes selectively a population of T lymphocytes participating in the primary response to Con A.

Besides theoretical interest, the observations have practical implications for studies of mitogen activation of human lymphocytes. Human blood lymphocytes purified by Ficoll–Isopaque or other sedimentation techniques without a step to remove adherent and phagocytic cells may contain much more than 10% monocytes (Boldt, Skinner & Komfeld, 1972, compare Fig. 4). Large variations of Con A-induced DNA synthesis are expected in such cultures mainly depending on their contents of monocytes.

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