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The effect of adherent and phagocytic cells on human lymphocyte PHA responsiveness

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SUMMARY

The effect of small numbers of adherent and phagocytic cells on the human peripheral blood lymphocyte response to PHA was examined by depleting these cells from lymphocyte preparations. Lymphocyte preparations obtained by centrifugation on Ficoll–Triosil, which contained on average 85% lymphocytes, responded well to PHA. Depletion of cells adhering to nylon fibre, giving a population containing on average 95% lymphocytes, resulted in a considerably reduced response. Depletion of cells that adhered to plastic or ingested iron powder to give populations containing on average 90% lymphocytes, also reduced the PHA response, but to a lesser extent. Reduction in PHA responsiveness correlated with increasing lymphocyte purity. The responsiveness of nylon-column-filtered cells could be restored by adding a small number of cells from a monocyte-rich population.

INTRODUCTION

Stimulation of lymphoid cell populations by mitogens such as PHA results in transformation of lymphocytes to blast cells in active DNA synthesis. It has been reported that highly purified lymphocyte populations respond poorly to mitogen stimulation and that the responsiveness can be restored by adding glass adherent leucocytes, probably of the monocyte-macrophage series (Levis & Robbins 1970; 1972).

It is now possible to identify two major populations of lymphocytes (T and B cells) by surface marker tests and it has been shown that some methods used for preparing highly purified lymphocyte populations such as nylon fibre column filtration also result in depletion of the B-lymphocyte population (Julius, Simpson & Hergenberg 1973; Brown & Greaves 1974). It is not clear therefore whether the reduced mitogen responsiveness is due to removal of non-lymphoid cells, B lymphocytes or cells of both types.

In the present study, lymphocyte-rich populations prepared from peripheral blood on a Ficoll–Triosil gradient were further depleted of adherent or phagocytic cells by several methods, in an attempt to dissociate the effect on PHA responsiveness of removing non-lymphoid cells and B lymphocytes. Lymphocyte-enriched populations were variously depleted of cells adhering to nylon fibre or plastic surfaces or of cells phagocytosing carbonyl iron. The T- and B-cell composition of these populations was also examined to determine to what extent the depletion procedure had altered the proportion of these cell types.

MATERIALS AND METHODS

Lymphocyte separation and nylon column filtration. Heparinized peripheral blood from healthy donors was initially separated by centrifugation on Ficoll–Triosil to yield a lymphocyte-rich population. This method together with the technique for depleting cells adherent to nylon fibre by column filtration has been described previously (Potter & Moore, 1975).

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Depletion of plastic adherent cells. Cells adhering to plastic surfaces were removed by incubating lymphocyte preparations (resuspended in Hanks's balanced salt solution (HBSS)+10% foetal calf serum (FCS)) in tissue culture flasks (Corning 25,100) for 1 hr at 37°C and the non-adherent cells collected. In some experiments the adherent cell population was also recovered by adding 0·02% EDTA and pipetting vigorously.

Depletion of phagocytic cells. Lymphocyte preparations obtained by Ficoll–Triosil centrifugation were depleted of phagocytic cells by incubating with carbonyl iron (BDH Limited) for 1 hr at 37°C and removing cells that had ingested iron using a magnet.

T- and B-cell estimation and differential cell counts. The proportions of T and B lymphocytes in the initial cell population and following the depletion procedures were determined by enumeration of rosette-forming cells and surface immunoglobulin (Ig)-bearing cells, as previously described (Potter & Moore, 1975). Differential cell counts were also performed using Jenner–Geimsa-stained cytocentrifuge preparations.

Monocyte-enriched populations. Monocyte-enriched populations were prepared by bovine serum albumin (BSA) gradient centrifugation, essentially as described by Holm & Hammarström (1973) and Brodersen & Burns (1973). Heparinized blood was first sedimented with dextran (20 ml of blood+4 ml of 3% dextran) and the resulting leucocyte population was washed in HBSS before being resuspended in a solution of BSA in HBSS (final concentration 27% in 2·6 ml). This solution was overlayed with 1·7 ml HBSS and centrifuged at 400 *g* for 40 min at room temperature. The cells accumulating at the interface were then carefully removed; this population contained an average 47±5% (s.e.) monocytes, 45±4% lymphocytes and 8±2% polymorphs.

PHA stimulation. Lymphocytes were cultured in Cooke microplates at a concentration of 2×10^5 cells per well in 0·2 ml of HEPES-buffered Minimum Essential Medium supplemented with 10% human AB serum. Wellcome purified PHA was added to give a concentration of 2 μg/ml which has been shown to produce optimal stimulation in our test system. All tests were set up in triplicate and unstimulated control cells were always included. Cultures were incubated for 3 days at 37°C in a humid chamber and 6 hr before the end of the culture period 1 μC of [^{3}H]thymidine (Amersham; specific activity 2·5 c/mMol) was added to each well in 50 μl of culture medium. At the end of the incubation time the cells were collected on fibre discs using a SAM 2 multiple culture harvester and after drying the discs were placed in vials containing scintillation fluid (toluene containing PPO and POPOP) for counting. The results were expressed as mean values of triplicate cultures in c.p.m.

RESULTS

The percentages of T and B lymphocytes were determined in lymphocyte preparations obtained by centrifugation on Ficoll–Triosil and also after further purification procedures (Fig. 1). Lymphocyte preparations were depleted of cells adhering to nylon fibre, cells adhering to plastic or cells ingesting iron powder.

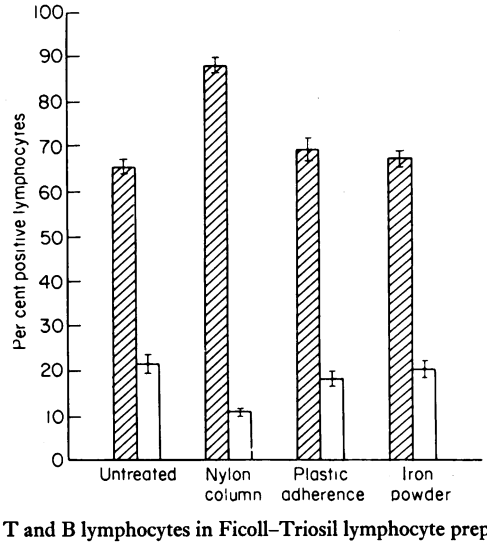

FIG. 1. The percentage of T and B lymphocytes in Ficoll–Triosil lymphocyte preparations further purified by nylon-column filtration, plastic adherence or iron powder treatment. The percentage of T cells was determined by E-rosette formation (hatched columns) and of B cells by fluorescent staining of surface immunoglobulin (open columns). The values represent the mean (±s.e.) of eight experiments.

In this series of experiments Ficoll–Triosil lymphocyte preparations contained on average 66±1% (SE) T lymphocytes as determined by E-rosette formation and 22±2% B cells as determined by fluorescent staining of surface Ig (Fig. 1). Following nylon column filtration the proportion of T lymphocytes was considerably increased to an average value of 88±1% rosette forming cells, while that of Ig^+ cells fell to 11±1%. Removal of cells ingesting iron powder did not significantly alter the proportion of T and B cells compared with non-depleted preparations, the proportions of rosette-forming cells and Ig^+ bearing cells being 67±2% and 20±2% respectively. Removal of plastic adherent cells produced a slight increase in the proportion of T cells present, viz. 70±3% E-rosette forming cells and 18±2% Ig-bearing cells.

TABLE 1. Summary of differential cell count and PHA responsiveness in Ficoll–Triosil (FT) preparations and preparations further purified by plastic adherence, iron-powder treatment or nylon column filtration.

Purification procedure	Percentage			PHA response as percentage of control
	Lymphocytes	Polymorphs	Monocytes	
FT only	85·2±2·0	10·5±1·6	4·4±0·8	100
FT+plastic adherence	89·6±1·4	6·8±0·8	3·6±0·7	73·6±7·4
FT+iron powder	90·1±1·3	7·1±1·2	2·8±0·3	76·3±9·1
FT+nylon column	95·0±0·6	3·7±0·5	1·4±0·4	45·8±8·1

PHA responsiveness is given relative to control cells separated by FT only. Each value represents the mean (±s.e.) of six to eight experiments.

Percentage lymphocytes in depleted populations

The percentages of lymphocytes in control and depleted Ficoll–Triosil preparations were also determined by examination of stained cytocentrifuge preparations (Table 1). The initial Ficoll–Triosil separation produced a population containing an average of 85% lymphocytes, 11% polymorphs and 4% monocytes. Nylon column filtration resulted in an increase in the percentage of lymphocytes to an average value of 95% with only 4% polymorphs and 1% monocytes. Populations depleted by plastic adherence or iron-powder treatment showed increases in the percentage of lymphocytes to 90% with 7% polymorphs and 3% monocytes.

PHA responsiveness of depleted populations

Ficoll–Triosil preparations of blood lymphocytes responded well to PHA although there was some variation in the size of the response with values of 10,000–22,000 ct/min and a mean value of 15,000 ct/min (Fig. 2). Following nylon column filtration the PHA response was considerably reduced in all cases when compared with the response produced by control cells not passed through the column. The response produced varied between 18 and 87% of the corresponding control value with a mean value of only 46%.

The PHA response of Ficoll–Triosil preparations depleted of cells by plastic adherence or ingestion of iron powder showed similar responses. The PHA response was reduced in all but one case (where iron powder treatment produced a 5% increase) with values of 35–97% of the response produced by control cells. However, the reductions were generally less than those seen following nylon column filtration, populations depleted by plastic adherence gave on average 74% of the control response, while populations depleted by iron powder treatment gave on average 76% of the control response (Fig. 2). All lymphocyte preparations gave low [^{3}H]thymidine incorporation when cultured without PHA (100–400 ct/min per culture).

When the percentage of lymphocytes in the depleted populations is compared with the PHA response produced (Table 1) it is observed that reduction in the response correlates with increased lymphocyte purity, suggesting a role for monocytes or polymorphs in PHA stimulation.

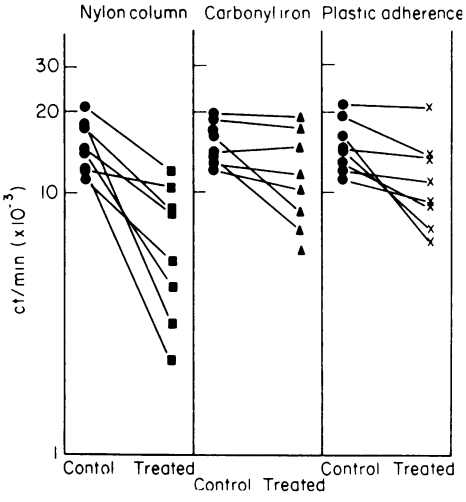

FIG. 2. PHA stimulation of Ficoll–Triosil lymphocyte preparations (●) and following further purification by nylon column filtration (■), plastic adherence (×) or iron powder treatment (▲). Equal numbers of lymphocytes from each preparation (2×10^5/culture) were cultured for 3 days with 2 μg/ml of PHA and [^{3}H]-thymidine incorporation was measured during the final 6 hr of culture. Results are expressed as the mean ct/min for triplicate cultures and each line represents a separate esperiment.

Monocyte-enriched populations were prepared by BSA gradient centrifugation, and the ability of these cells to restore the PHA response of nylon column filtered cells from the same individual was examined. Nylon-column-filtered cells were stimulated by PHA and the response produced was measured with and without the addition of a small number of cells from the monocyte preparation (2×10^5 nylon column cells+2×10^4 BSA gradient cells). The PHA response of this number of BSA gradient cells alone was also determined as well as the response of control cells plus BSA gradient cells. It was found that addition of BSA gradient cells restored the PHA response produced by nylon filtered cells to the level produced by control cells (Fig. 3). BSA gradient cells alone produced virtually no response and when added to control cells evoked only a marginal increase in responsiveness, suggesting that the number of cells added could not themselves produce the increased PHA response observed with nylon column filtered cells.

In further experiments it was found that the PHA responsiveness of lymphocyte preparations depleted

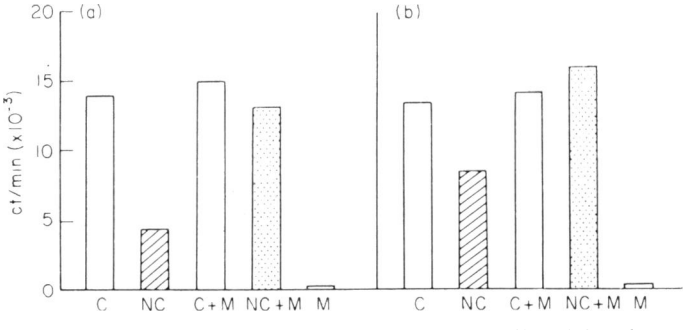

FIG. 3. Reconstitution of the PHA responsiveness of nylon-column-filtered lymphocytes by adding a monocyte-enriched population prepared by BSA gradient centrifugation. Nylon column (NC) and control (C) lymphocyte populations (2×10^5/culture) were stimulated by PHA (2 μg/ml) with and without the addition of 2×10^4 cells of a monocyte-enriched population (M). Monocytes alone were also cultured with PHA. [^{3}H]-thymidine incorporation was measured after 3 days of culture and the results represent the mean values of triplicate cultures in two experiments (a and b).

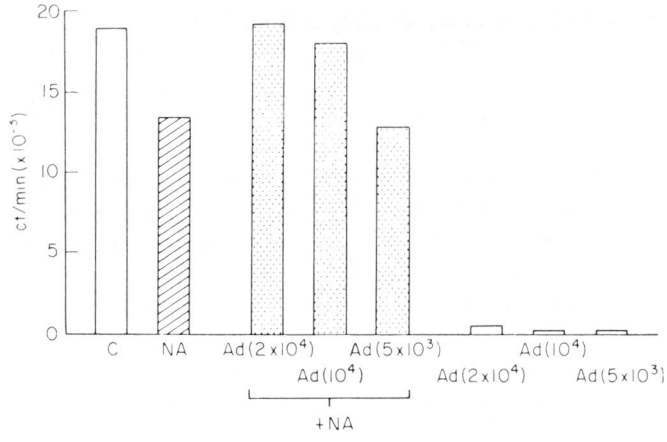

FIG. 4. Reconstitution of a lymphocyte preparation depleted of plastic adherent cells by adding plastic adherent cells removed by treatment with EDTA. Nonadherent (NA) and control (C) lymphocyte populations (2×10^5/culture) were stimulated by PHA with and without the addition of adherent cells (Ad) (2×10^4, 10^4 or 5×10^3 per culture). Adherent cells alone were also cultured with PHA. [^{3}H]thymidine incorporation was measured after 3 days of culture and the results represent the mean values of triplicate cultures.

by plastic adherence could be restored by adding back plastic adherent cells removed by treatment with EDTA (Fig. 4). The PHA response of 2×10^5 cells of the depleted population could be restored to control values by adding 2×10^4 or 10^4 adherent cells but 5×10^3 adherent cells had no effect.

DISCUSSION

There is much evidence to suggest that the principal responding cell in lymphocyte preparations stimulated by PHA is the T lymphocyte (Greaves & Janossy, 1972) but in earlier studies we reported that highly purified T-cell populations from human blood responded poorly to PHA, suggesting that small numbers of accessory cells may be required for optimum stimulation (Potter & Moore, 1975).

In the present study the effect of small numbers of phagocytic cells (monocytes and polymorphs) on the lymphocyte response to PHA was further examined by removal of these cells from lymphocyte preparations and also reconstitution of depleted populations by the addition of cells from a monocyte-enriched population.

Ficoll–Triosil gradient separation of peripheral blood produced a lymphocyte-rich cell preparation which responded well to PHA stimulation, but these preparations still contained a significant number of residual monocytes and polymorphs (combined average value 15%). Additional nylon column filtration produced a depletion of the monocyte and polymorph populations (to about 1 and 4% respectively) as well as a depletion of the B-lymphocyte population; these preparations had a reduced PHA response. Depletion of plastic adherent cells or cells ingesting iron powder, two procedures which reduce the monocyte and polymorph composition without substantially altering the T:B cell ratio, also diminished the PHA response but to a lesser extent than nylon column filtration. Reduction in PHA responsiveness appeared to correlate with the depletion of phagocytic cells since nylon column filtration produced the most efficient removal of these cells and also the largest reduction in PHA response. Plastic adherence or iron powder treatment produced a smaller reduction in PHA response corresponding to less efficient elimination of phagocytic cells.

The PHA responsiveness of nylon-fibre-column-filtered cells could be restored by adding a small number of cells from a monocyte-enriched population. This population, prepared by BSA gradient centrifugation contained on average 47% monocytes and 45% lymphocytes with very few polymorphs, indicating that if polymorphs have any role at all in the restoration of the PHA responsiveness of depleted populations, it is secondary to that of monocytes. Our depletion and reconstitution experiments suggest that the number of monocytes required for optimum lymphocyte stimulation by PHA is of the

order of 2–5% but it does not rule out the possibility that small numbers of B cells may also enhance the PHA response. The mechanism by which monocytes enhance the PHA response is still unknown, but since PHA can bind directly to lymphocyte surface membranes, one possible explanation is that monocytes are required for the survival of stimulated lymphocytes in culture, perhaps by virtue of a conditioning effect.

Other workers have reported reduced mitogen responses in lymphocyte populations depleted of adherent cells. Levis & Robbins (1970; 1972) depleted leucocyte preparations (obtained by gravity sedimentation) by passage through nylon fibre columns and obtained a decreased PHA response which could be restored by adding glass adherent cells. However, at the time of this study surface marker tests were not available for monitoring the proportion of T and B cells present. Hedfors *et al.* (1975) reported that Con A-stimulation of purified T cells is monocyte dependent, but in this system optimum stimulation required a larger number of monocytes (1:1 lymphocyte:monocyte ratio). In our study increasing the percentage of monocytes present by addition of a monocyte-enriched population to Ficoll–Triosil separated cells produced only a marginal increase in PHA responsiveness, but this experiment was not taken to equivalence in respect of the lymphocyte–monocyte components. Using rat lymph node cells Folch *et al.* (1973) obtained a reduced response to low doses of PHA following depletion on a glass bead column which could be restored by the addition of small numbers of macrophages (0·25 and 1%); higher macrophage numbers (5%) depressed the response. Also, Keller (1975) using rat spleen cells depleted of plastic-adherent cells obtained an increased response to Con A in the presence of small numbers of added macrophages (1:10 and 1:25 macrophage:spleen cell ratio) whereas large numbers reduced the response (1:1 ratio). Several studies in human and experimental animal systems thus reveal evidence of a direct participation of monocytic cells in PHA responsiveness, although their effects are critically dependent on the numbers present and the source from which they are derived.

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