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Lymphocyte responses to phytohaemagglutinin: age-related effects

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Summary. Cell-mediated immunity is depressed in elderly individuals compared to young individuals, and lymphocytes from elderly individuals have been reported to have impaired lymphocyte responsiveness to stimulation by PHA after 4 days of culture. We have confirmed this observation. However, after 8 days of culture, the lymphocyte responses were greater in elderly normal individuals than in young normal individuals. Responses of lymphocytes from young individuals decreased with time from 4 to 8 days in culture, while there were increased responses with time when lymphocytes from elderly individuals were studied. When adherent cells from lymphocytes of young individuals were removed by passage through protein-coated Degalan-bead columns, the lymphocyte responses to PHA were significantly increased at 8 days. Passage of lymphocytes from elderly individuals through coated Degalan bead columns did not alter the lymphocyte responses. Removal of macrophages from the mononuclear cells obtained from young individuals did not result in increased lymphocyte responses to PHA after 8 days in culture. Removal of adherent cells appeared to have the same effect regardless of the efficiency of removing B cells. The adherent cells removed by the protein coated columns, therefore, appear to be nonphagocytic mononuclear cells which are not B lymphocytes.

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INTRODUCTION

There is an increased incidence of neoplasm (Dorn & Cutler, 1959) and serum autoantibodies in older human individuals as compared to young individuals (Allison, Denman & Barnes 1971). These observations may relate to reports of decreased immune responsiveness on the basis of impaired T-cell function in the aged (Roberts-Thompson, Whittingham, YoungChaiyud & MacKay, 1974). An *in vitro* parameter of T-cell function is the response of lymphocytes to stimulation with phytohaemagglutinin (PHA). Lymphocytes from elderly subjects have been reported to have impaired responsiveness to stimulation by PHA (Fernandez, MacSween & Langley, 1974; Weksler & Hutteroth, 1974; Foad, Adams, Yamauchi & Litwin, 1974). Our investigations suggest that this difference is not a simple one.

We and others reported previously that responses of unseparated lymphocytes from young normal individuals to stimulation by PHA was greater than responses of lymphocytes from old normal individuals after 4 days of *in vitro* culture (Fernandez *et* al., 1974; Foad *et al.*, 1974; Fernandez, MacSween & Langley, 1976). However, in our studies, after 8 days of incubation, the responses of lymphocytes from younger individuals were significantly less than older individuals. After passage of lymphocytes through antigen–antibody-coated Degalan bead columns, the pattern of lymphocyte responses to

stimulation by PHA in the old individuals was unchanged. However, the pattern of response in the younger individuals was reversed, responses being greater at day 8 than at day 4. This observation raised the possibility that reduced responsiveness of lymphocytes from young individuals after 8 days of culture was due to an adherent cell type which was removed by passage through the Degalan-bead columns.

MATERIALS AND METHODS

Heparinized blood was obtained from young individuals, less than 40 years of age, and old normal individuals, greater than 65 years of age. Mononuclear cells were obtained by the ficoll–isopaque density gradient method (Böyum, 1968). B-cell numbers were estimated by the presence of membrane-bound IgM by immunofluorescence (Fernandez, MacSween & Langley, 1975). T cells were identified by their ability to bind spontaneously to SRBC (Brain, Gordon & Willet, 1970). Monocytes were identified by staining the peripheral blood with Wright's Geimsa strain.

Monocytes from the peripheral blood were removed as described by Lundgren, Zukoski & Moller, 1968. Briefly, 15–20 million mononuclear cells per 2 ml of Medium 199 was added to 2 ml of an isotonic suspension of carbonyl iron and 0·2 cc of heat-inactivated foetal calf serum, and rotated at 37° for $1\frac{1}{2}$ h in 5×1-cm tubes. After this time, a magnet was attached to the side of the tubes and the tubes further rotated for another 5 min. The iron filings and the monocytes containing iron filings remained attached to the tubes near the poles of the magnet. The rest of the mononuclear cells were removed, washed three times with Medium 199, and placed in culture.

B cells were removed by passage of lymphocytes through Degalan-bead columns coated with IgG-anti-IgG as described previously (Fernandez *et al.*, 1975). Briefly, Degalan beads were coated with human IgG and poured into a Pasteur pipette and then complexed with rabbit anti-human IgG. The beads were washed with excess Medium 199. Twenty to twenty-five million peripheral blood mononuclear cells were placed on the column for 2 h at 4°. The cells were then eluted with Medium 199. B cells were effectively depleted from the mononuclear suspension. Degalan beads were similarly coated with 3 per cent bovine serum albumin (BSA) and were also used as uncoated beads. The mean percentages of mononuclear cells eluted from IgG anti-IgG columns, BSA-coated columns and uncoated columns were 50·6, 46·6 and 23·8 respectively.

Triplicate samples of 200,000 cells were placed in 15-ml plastic culture tubes with 3 ml of Medium 199 containing 15 per cent foetal calf serum, penicillin 100 u/ml and streptomycin 100 μg/ml. 1·0 mg of PHA was added to the lymphocyte suspensions being tested. The tubes were incubated at 37° in 5 per cent carbon dioxide and air. Tritiated thymidine, 0·5 μCi, was added to each tube on either the third or seventh day. The cultures were harvested on the following day, and the radioactivity incorporated by the lymphocytes was determined as described by Hughes & Caspary (1970).

RESULTS

B and T cells in the peripheral blood

The mean percentage of B cells in young normal individuals as estimated by immunofluorescence was $15{\cdot}5 \pm 1{\cdot}1$ (s.e.m.) and in old normal individuals was $12{\cdot}3 \pm 1{\cdot}0$ ($P < 0{\cdot}05$). There was no significant difference in the percentage of T cells in young normal individuals, $45{\cdot}9 \pm 5$, as compared to old normal individuals, $46{\cdot}6 \pm 7{\cdot}0$.

Lymphocyte responses to PHA

Unseparated and separated lymphocytes by antigen–antibody columns

The unseparated lymphocyte responses to stimulation by PHA were greater in twenty-four young normal individuals as compared to twelve old normal individuals at day 4 ($P < 0{\cdot}05$). However, at day 8, responses were less in the young normal individuals as compared to old normal individuals (Fig. 1). When lymphocytes from young and old normal individuals were passed through Degalan bead columns coated with IgG anti-IgG complexes, the IgM-bearing cells were effectively reduced to less than 2 per cent in all cases. The T cells, from young normal individuals were increased to 59·4 per cent $\pm 7{\cdot}2$ and from old normal individuals to 59·7 per cent $\pm 6{\cdot}0$. The responses of the eluted cells to PHA in both groups showed a greater degree of stimulation at day 8 than at day 4 ($P < 0{\cdot}01$). The lymphocyte responses to stimulation by PHA in old

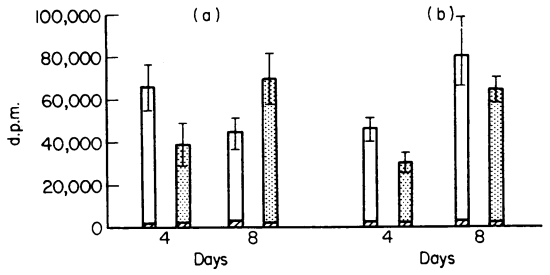

Figure 1. Lymphocyte responses to stimulation by PHA. (a) Unseparated; (b) separated. Open part of columns: d.p.m. in stimulated cultures from young individuals; stippled part of columns: d.p.m. in stimulated cultures from old individuals; hatched part of columns: d.p.m. in unstimulated cultures.

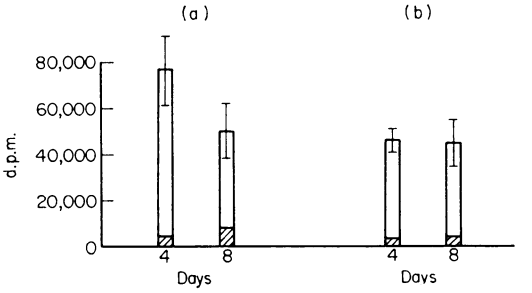

Figure 2. Lymphocyte responses to stimulation by PHA from young individuals. (a) Whole mononuclear population; (b) monocyte-depleted mononuclear population. Open part of columns: d.p.m. of stimulated cultures; hatched part of columns: d.p.m. of unstimulated cultures.

normal individuals were not significantly different in the separated as compared to the unseparated populations. However, at day 8, the responses of separated lymphocytes from young normal individuals was significantly greater than the unseparated lymphocyte population ($P < 0{\cdot}01$). Since this phenomenon was observed in the young normal individuals, the following experiments were performed on lymphocytes from young normal individuals only.

Removal of monocytes

The mean percentage of monocytes present in the peripheral blood was 9 per cent with a range of 8–14 per cent. After iron filing phagocytosis and removal by a magnet, the monocyte population was reduced to less than 1 per cent. The lymphocyte responses to stimulation by PHA were significantly less at day 4 than the whole mononuclear population. However, at day 8, no significant increases in stimulation were seen in the monocyte-depleted population, as compared to the whole mononuclear population, although increases in stimulation occurred in two of seven cases studied (Fig. 2).

Effect of passage of lymphocytes through uncoated and BSA-coated columns

The responses at day 4 and 8 of lymphocytes to stimulation by PHA after passage through uncoated columns was less than the response of unseparated lymphocytes, but the difference was not statistically significant.

Passage of whole lymphocyte populations through BSA-coated columns did not effectively deplete B cells. Twenty-five to 50 per cent of the original proportion of B cells placed on the column were still present in the eluted cells. T cells were increased to 48·6 per cent from 41·1 per cent prior to application to the column. However, in six of seven cases, the lymphocyte responses to stimulation by PHA were increased at day 8 as compared to day 4 ($P < 0{\cdot}05$). In the seventh case, the rise was equivocal (Fig. 3).

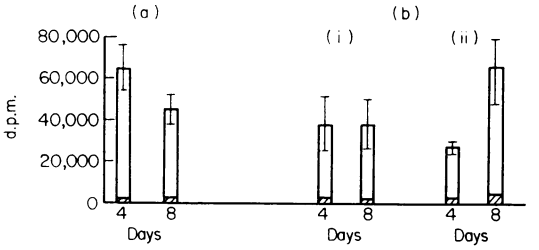

Figure 3. Lymphocyte responses to stimulation by PHA from young individuals. (a) Unseparated; (b) separated by (i) uncoated columns; (ii) B.S.A.-coated columns. Hatched part of column: d.p.m. of unstimulated cultures; open part of column = d.p.m. of stimulated cultures.

DISCUSSION

A decline in cellular immunity with age has been reported in animals. Responses of mouse spleen cells to stimulation by mitogens such as PHA, PWM, Con A and PPD, all show a striking decline with advancing age (Gerbase-Delima, Meredith & Walford, 1975). Spleen cells from old mice show a reduced capacity to elicit a GVH reaction (Peterson, 1972). Old mice show less cell-mediated lympho-

cytotoxic reactions and are less able to reject transplanted tumour cells (Teller, 1972).

Folch, Yoshinaga & Waksman, 1973 have reported that when whole mononuclear populations from the spleen of young rats are put through nylon-wool columns, the eluted cells have a greater response to stimulation by PHA than the whole cell populations. In the old rat, the response to stimulation is unchanged by passage of spleen cells through the column. They have also shown that the phenomenon which occurs in the young rats is due to the removal of an adherent thymus-dependent cell which inhibits PHA responses (Folch & Waksman, 1974). With advancing age, the inhibitory effect mediated by these adherent T cells disappears. There is also evidence that the inhibitory effect is mediated by soluble factors elaborated by the adherent cells (Dutton & Scavalli, 1975).

In our experiments, responses to PHA after 4 days of culture were reduced by removal of adherent cells on the columns. This is consistent with reports of the requirement of macrophages or monocytes for optimal responses to PHA (Gery, Gershon & Waksman, 1972). However, when cells from younger individuals were left for a longer period in culture (8 days), the responses of column-separated lymphocytes to PHA were significantly greater than the unseparated mononuclear cells. This is consistent with either enrichment of PHA-responsive cells by column separation or with the accumulation of inhibitory substances with time in the tubes containing unseparated cells. We feel that the former interpretation is unlikely in that column separation increased E-rosette forming cells by only 19 per cent. The latter interpretation is consistent with observations on rat lymphoid cells as noted above. Other interpretations for reduced responsiveness of unseparated cells at day 8 seem less likely. Exhaustion of cells by the initial response to stimulation seems unlikely in that the cells are capable of considerably greater responsiveness in autologous serum. This would also argue against the accumulation of toxic factors or utilization of nutrients, particularly as the cell density was very low. A shift in the optimal concentration of PHA for maximum stimulation is possible, but would have to be within relatively narrow limits as 0·1 mg of PHA was found to be suboptimal and 3 mg was toxic.

The nature of adherent cells removed by the column was further investigated by determining if depletion of either monocytes or B lymphocytes was necessary for increased stimulation at 8 days. Depletion of monocytes with iron filings reduced PHA responses at day 4. This has been reported by others and suggests that monocytes do enhance lymphocyte responses to PHA stimulation at this time period. No increase in lymphocyte responses to PHA at day 8 was seen after monocyte depletion, suggesting that these cells were not responsible for diminished responses at day 8. However, our data does not exclude the possibility that non-phagocyte precursor cells differentiate *in vitro* to form functional monocytes at day 8.

The necessity for B-cell depletion was investigated by passing the cells through uncoated Degalan-bead columns and through columns coated with a protein other than an anti-immunoglobulin (BSA). No change in responsiveness was seen after passage of cells through uncoated columns, but there was increased responsiveness to PHA of cells passed through columns coated with BSA after 8 days in culture. Since B cells were not effectively depleted by the BSA-coated columns, we concluded that they are not critical for this effect. The putative inhibitory cell, therefore, appears to be a non-phagocytic, non-B-cell lymphocyte which is weakly adherent in that it adheres to protein coated beads, but not to uncoated beads. This is consistent with an adherent T cell, although the possibility that it is a K cell cannot be discounted.

The nature of the inhibitory effect has not been elucidated. The possibility that it involves loss of viability or cell numbers has not been explored. Its significance *in vivo* is also not apparent, as this effect is not seen when cells are cultured in autologous or human AB serum. However, it has obvious implications in interpreting PHA responsiveness *in vitro* and points to an age-related difference in human lymphocytes which warrants further investigation.

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