Phytohemagglutinin-Induced Transformation and Aggregation of Guinea Pig Lymphocytes[1] (34302)

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Mitogenic agents which cause a high percentage of blast transformation when added to short-term lymphocyte cultures also cause marked cell aggregation. Conditions which promote cell-to-cell contact may increase the blastogenic reaction as evidenced in antigen-stimulated lymphocyte cultures (1, 2). Removal of those cells capable of adhering to glass eliminates the transformation seen in antigen-stimulated cultures (1–3). Despite the evidence that cell contact is an important aspect of response to specific antigens, the relationship of marked aggregation seen with phytohemagglutinin (PHA) and other nonspecific mitogens to blastogenesis is less clear. Lymphocytes purified by the glass bead method still transform when treated with PHA (1, 2). Coulson (4) has observed PHA-induced transformation even when cells are embedded in agar prior to treatment so that cell contact is eliminated. In the agar system it would seem difficult to measure the magnitude of this response. In the present work, the response of guinea pig lymph node lymphocytes to PHA is related to the aggregation characteristics of the treated cells.

Materials and Methods. Hartley strain inbred guinea pigs (Camm Research Institute, Inc., Wayne, N.J.) 300–350 g males were used in all experiments. These animals were immunized by subcutaneous injection into each hind footpad, 0.2 ml of alum-precipitated DPT antigen (Tri-Solgen, Eli Lily & Co.) at 10-day intervals. Ten to 20 days after the second immunization, the animals were sacrificed. Under aseptic conditions, popliteal lymph nodes were collected in TC 199 (Difco) with 20% fetal calf serum, gently minced, and expressed through a 100-gauge stainless steel wire mesh. This yielded a 98% single cell suspension which was washed twice and adjusted to a concentration of 4×10^6 cells/ml. A 0.5-ml portion of this suspension was then added to 1.5 ml of TC 199, with or without PHA (control), and cultured in vertical, tightly stoppered 15-ml round bottom Kimax tubes at 37° in 5% CO_2–air. Cells were routinely harvested at 1, 48, and 96 hr.

The PHA used in all experiments was from a lot prepared in this laboratory by the method of Börjeson *et al.* (5), contained 2.8 mg of protein/ml, and was stored frozen at —20°. A new vial was opened for each experiment. The optimum dose of PHA for a culture containing 1×10^6 cells/ml was 0.001 ml (2.8 μg of protein) /ml of culture.

Tritiated thymidine incorporation was determined by the following procedure: 16 hr prior to harvest, 1 μCi of thymidine-methyl-(^{3}H), 15.9 Ci/mmole (New England Nuclear Corp., Boston, Mass.) was added to each tube to be assayed. At harvest, tubes were cooled in ice and washed three times with 5 ml of iced 0.9% saline. Between washes the cells were centrifuged quickly at 2000*g* for 5 min and decanted at 4° by vertical drainage. A 0.5-ml portion of NCS, (Nuclear Chicago, Des Plaines, Ill.) was added to each cell button and incubated for 30 min at 56°. The digestion mixture was then transferred to 10.0 ml of Bray's scintillant and counted in a liquid scintillation counter to a statistical error of 1.5%. Results were expressed as counts per minute per 10^8 μ^3 cell mass

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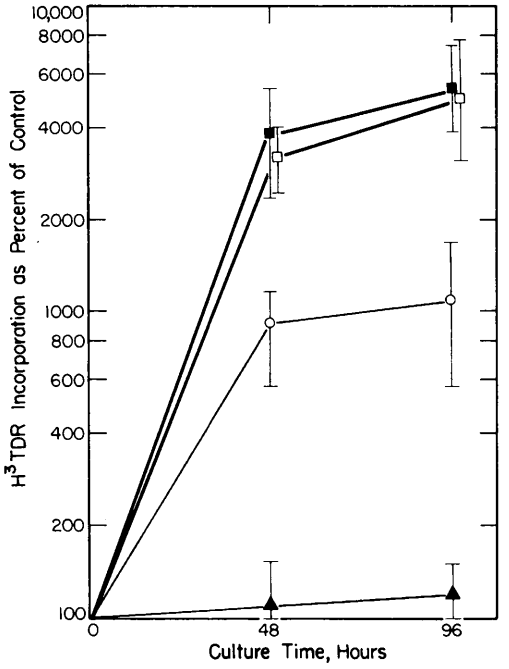

FIG. 1. Culture growth rate using different concentrations of PHA per ml of culture as measured by thymidine-(^{3}H) incorporation: 0.00025 ml of PHA (○); 0.001 ml of PHA (■); 0.005 ml of PHA (□); 0.10 ml of PHA (▲); vertical bars represent ±1 standard error from 5 experiments.

present at the time of harvest after corrections for quenching, as determined by internal standardization, and counting efficiency.

Pronase–cetrimide cell counts and nuclear sizing were done by the method of Stewart and Ingram (6). At harvest, 0.8-ml aliquots of cells were incubated at 37° for 20 min with 0.8 ml of the proteolytic enzyme pronase, 5 mg/ml (Cal Biochem, Los Angeles, Calif.). To the incubation mixture, 38.4 ml of a counting solution containing the detergent cetrimide, 5 mg/ml (hexadecyltrimethylammonium bromide, Eastman Organic Chemicals, Rochester, N. Y.) was added. Viable cell counts and nuclear sizing were completed within 15 min using the electronic particle counter beginning at a threshold of 12 μ^3 and increasing to 300 μ^3.

Cell sizing was accomplished in a similar manner, using the original cell suspensions at harvest, diluted 1:50 in 0.9% saline and counting over a volume range of 60–30,000 μ^3 with the electronic particle counter and 100-μ orifice, calibrated with ragweed and pecan spores. Total culture mass was calculated from these data using a program developed for the G E no. 600 Series computer in BASIC (7).

Cell differentials obtained at harvest were prepared by smearing a concentrated cell suspension on glass slides with a smooth-edged cover glass, drying immediately with warm air, and staining by the May-Gruenwald Giemsa method. Two observers performed 1000 cell counts, each, to determine the percentage of cells in aggregates and mean number of cells per aggregate.

Results. Figures 1, 2, and 3 reflect the growth rate, measured by thymidine-(^{3}H) incorporation, and total growth, measured by nuclear sizing and total cell mass of PHA-

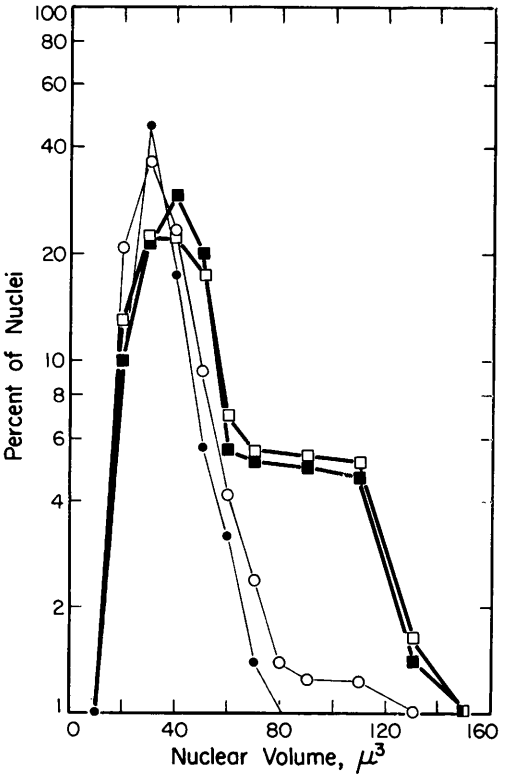

FIG. 2. Mean nuclear volume distribution patterns of pronase–cetrimide–treated cells after 48 hr of culture: No treatment (●); 0.00025 ml of PHA/ml of culture (○); 0.001 ml of PHA/ml of culture (■); 0.005 ml of PHA/ml of culture (□).

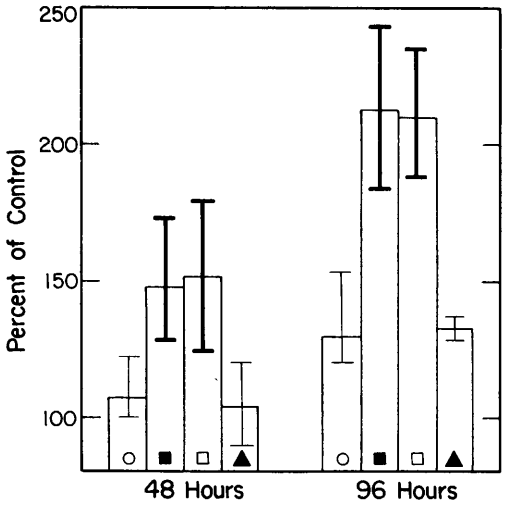

FIG. 3. Percentage change in total cell mass at different concentrations of PHA per ml of cultured cells compared to control cells tested at the same time: 0.00025 ml of PHA (○); 0.001 ml of PHA (■); 0.005 ml of PHA (□); 0.10 ml of PHA (▲); vertical bars represent ±1 standard error from 5 experiments.

treated guinea pig lymphocytes. These are compared to untreated cultures at the same periods of observation. Data regarding transformation and aggregation of guinea pig lymph node lymphocytes have been presented elsewhere (7). Low (0.00025 ml of PHA/ml of culture) and high (0.10 ml of PHA/ml of culture) PHA concentrations result in a suboptimal response, while stimulation with 0.001 ml of PHA/ml of culture or five times this amount produces a significantly greater and equal response.

We have previously defined three divisions of PHA-treated guinea pig lymphocyte aggregate distribution, as measured with the electronic particle counter (7). *Division I*, that volume range encompassing the majority of unstimulated cells as determined by sizing of control cultures, (60–300μ^3); *Division II*, that volume range including large single cells (diameter $>$ 8.4 μ) and small aggregates (300–3000 μ^3); and *Division III*, that group of particles greater than 3000 μ^3. With PHA treatment, there is a shift in the distribution of particles within these divisions (Fig. 4), indicating that although by 48 hr cells treated with 0.001 and 0.005 ml of PHA/ml

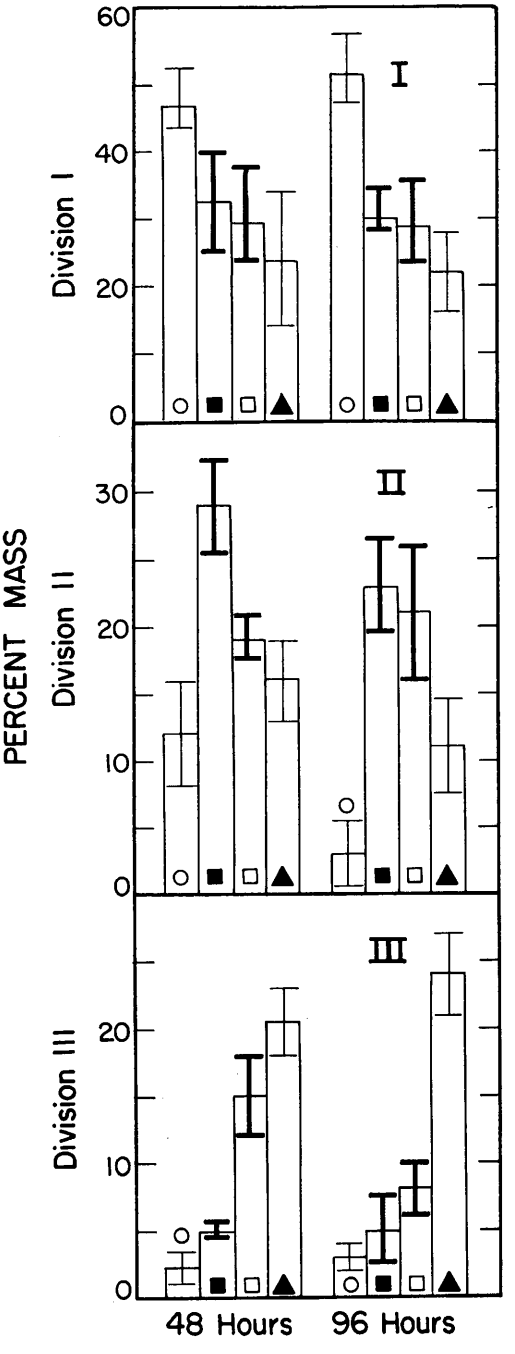

FIG. 4. Redistribution of cell mass between Divisions I, II, and III at 48 and 96 hr with different concentrations of PHA per ml culture. 0.00025 ml of PHA (○); 0.001 ml of PHA (■); 0.005 ml of PHA (□); 0.10 ml of PHA (▲); vertical bars represent ±1 standard error from 5 experiments.

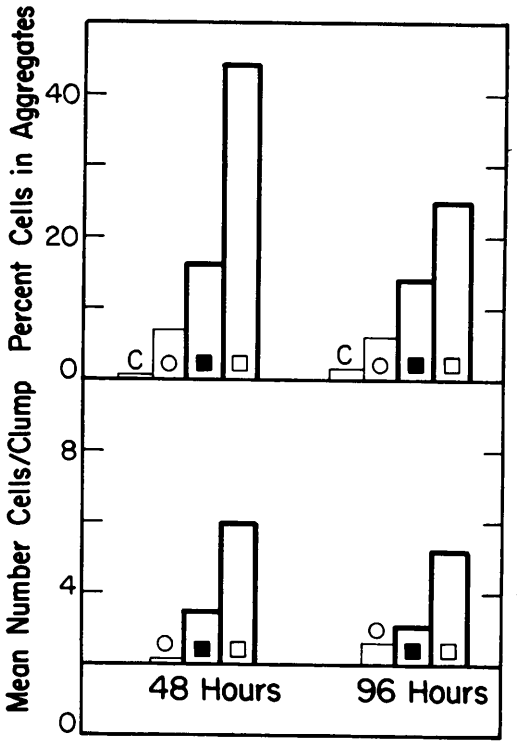

FIG. 5. Comparison of cell aggregation at different concentrations of PHA per ml of culture at 48 and 96 hr, as quantitated by stained smears, illustrating the percentage of cells in aggregates and the mean number of cells per aggregate: untreated cells (C); 0.00025 ml of PHA (○); 0.001 ml of PHA (■); 0.005 ml of PHA (□).

of culture have left Division I to the same degree, the former group of cells are found predominantly in Division II and the latter group in Division III. By 96 hr this difference is not significant. Examination of stained preparations (Fig. 5), a less reliable means of assessing aggregation since the technique allows an unequal distribution of particles, also indicates approximately a twofold increase in aggregation of cells treated with 0.005 ml of PHA/ml of culture as compared to those treated with 0.001 ml of PHA/ml of culture.

Discussion. Lymphocyte transformation and aggregation are PHA dose dependent although maximum transformation occurs at an optimal PHA dose above which DNA synthesis declines (8–10). It has been suggested that the latter decline in growth is a result of PHA cytotoxicity reflected by marked cell aggregation (11), and decreased viability (12–14). Although hemagglutinating and mitogenic properties of PHA have been separated (15), no such separation has yet been demonstrated between the leukoagglutinating and mitogenic properties of PHA (16). Transformation of single lymphocytes exposed to PHA while embedded in agar suggests that these two properties may also be separate (4). The present studies indicate that a fivefold increase in the optimum PHA dose fails to alter the growth characteristics of lymphocytes, as measured by DNA synthesis, total cell mass and nuclear size distribution, but causes almost a twofold increase in cell aggregation as measured by volume compartment change, percentage of aggregated cells and mean cells per aggregate. A hundredfold increase in optimum PHA dose results in further aggregation but a marked decrease in DNA synthesis.

It is therefore suggested that in the PHA dose range where growth is constant, either the properties of transformation and aggregation are independent, or after the optimum amount of cell surface contact is achieved, further increments of PHA are not beneficial and may eventually depress transformation by direct cytotoxicity or by overaggregation.

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