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Anchorage and lymphocyte function

II. CONTACT WITH NON-CELLULAR SURFACES, CELL DENSITY AND T-CELL ACTIVATION

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Summary. Varying numbers of human blood lymphocytes were stimulated by concanavalin A (Con-A) in a constant medium volume. At 'low' cell densities DNA synthesis was proportional to the number of cells in the cultures whereas at 'high' densities DNA synthesis was considerably lower than expected. In the presence of non-mitogenic microcarrier beads, (diameter 195 μm) to which the cells attached, DNA synthesis was proportional or nearly proportional to the cell number in the cultures also at 'high' cell densities. The potentiating effect of beads on lymphocyte stimulation was particularly noteworthy in individuals showing a weak mitogen response. Another approach that yielded proportionality between DNA synthesis and cell number both at 'low' and 'high' cell densities was the use of culture vessels with a larger bottom area. Under such conditions the presence of beads did not augment DNA synthesis.

These results suggest that the availability of a non-cellular adhesive surface is a major limiting factor and cell density a major regulating factor in the control of lymphocyte activation. Anchorage of the cells to a surface may modulate the density dependent 'growth control mechanism' indirectly via an influence on cell–cell interaction. An alternative less-likely interpretation is that the contact with non-cellular surfaces directly gives rise to regulatory responses in lymphocytes or accessory cells.

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INTRODUCTION

The growth of normal fibroblasts and epithelial cells is anchorage-dependent, i.e. the cells are unable to grow unless attached to a substratum (Stoker, O'Neill, Berryman & Waxman, 1968). Lymphocytes and other haematopoetic cells grow well in suspension culture and lack this anchorage dependence. Anchorage-dependent cells gradually stop DNA synthesis as the cells become confluent (Zetterberg & Auer, 1970). This decrease in DNA synthesis associated with cell crowding is termed density-dependent inhibition of growth (Stoker & Rubin, 1967).

The initiation of growth of normal T lymphocytes by lectin mitogens such as concanavalin A (Con-A), seems to require the presence of accessory cells of the monocyte-macrophage series (Levis & Robbins, 1970; Schmidtke & Hatfield, 1976; Habu & Raff, 1977). The mitogen-triggered activation process leads to the formation of growth factors that make activated cells go through the mitotic cycle (Gillis & Smith, 1977; Larsson & Coutinho, 1979). The mitogen-induced transformation of lymphocytes has been proposed to

be strongly dependent on cell–cell contact, a concept which is supported by several observations. Accordingly, lectins produce agglutination. Increased cell concentrations and culture conditions that optimize intercellular contact enhance stimulation (Moorhead, Connolly & McFarland, 1967; Hersh & Harris, 1968; Peters, 1972). Lectin-induced lymphocyte stimulation has been reported to be strongly dependent on cell density and to occur exclusively in cell aggregates (Peters, 1972). Stimulation of lymphocyte transformation by the oxidizing agents periodate and galactose oxidase requires intercellular contact (O'Brien, Parker & Dixon, 1978). This seems to indicate that a high cell density, even aggregation, is optimal for lymphocyte growth, thus distinguishing it from that of anchorage-dependent cells. However, we show here that high cell density inhibits Con-A-induced stimulation of human lymphocytes to DNA synthesis. This density-dependent inhibition can be abolished by an increase in substrate-surface area while the volume of culture medium is kept constant. The present study was initiated by our previous observations that certain lectins can provoke spreading of human lymphocytes on adhesive surfaces and that contact with solid surfaces augments T-cell activation (Wanger & Sundqvist, 1980; Sundqvist & Wanger, 1980).

MATERIALS AND METHODS

Cell purification

Mononuclear cells were isolated from defibrinated venous blood from healthy adults by sedimentation in gelatin. These cells were then incubated with iron powder (4 mg/10^6 lymphocytes) at 37° for 30 min and phagocytic cells and remaining iron powder were removed by a magnet. Erythrocytes were lysed by addition of ammonium chloride (0·83%). Separation of the mononuclear cells into T-lymphocyte enriched and depleted fractions respectively was performed using sheep-erythrocyte rosette sedimentation as described earlier (Natvig, Perlman & Wigzell, 1976). The viability of the purified lymphocytes was generally greater than 98%.

Cell culture and substrates

Stimulation of lymphocytes by Con-A was performed using triplicate cultures of varying numbers of mononuclear cells in 1 or 2 ml RPMI 1640 (Gibco) with 10% human AB serum in round-bottomed 10 × 75 mm glass tubes or in petri dishes (Falcon 3001 or 1008). Con-A was obtained from Pharmacia Chemicals, Uppsala, Sweden. The mononuclear cells used were stimulated by Con-A in the presence and absence of microcarrier beads of DEAE-Sephadex purchased from Pharmacia, Uppsala, Sweden (Cytodex). The wet mean size of these beads swollen in phosphate-buffered saline (PBS) was 195 μm. Beads (10^4) were added to each cell culture. The culture period was terminated with an 18-hr pulse of 0·2 μCi [^{14}C]-thymidine per ml culture medium. DNA synthesis was measured as incorporation into the trichloroacetic acid insoluble fraction of the cells.

Microscopy

The interaction of mononuclear cells with the surface of the beads was examined in a Leitz Orthoplan microscope with interference contrast Normarski optics. The photographs of this interaction were however taken in a Leitz Dialux microscope equipped with an automatic camera using Kodak tri-x film.

RESULTS

Bead-induced augmentation of lymphocyte stimulation related to cell density and the bottom area of the culture vessel

Increasing numbers of mononuclear cells, in a constant medium volume in round-bottomed tubes, were stimulated by Con-A in the absence or presence of Cytodex either for varying time periods or 3 days, the time when the stimulation in the absence of Cytodex was maximal. Figure 1 shows thymidine incorporation versus the cell number in the cultures on day 3 with cells obtained from four different individuals. In three of these (Fig. 1a,b,c) the dose–response curves in the absence of beads consist of two parts, one corresponding to cell densities below 10^6 cells per ml, and the other, corresponding to 10^6 cells per ml and higher densities. Below 10^6 cells per ml the magnitude of the stimulation was proportional to the cell number whereas it was markedly lower than expected at higher cell densities. In the individuals studied in Fig. 1a,b,c the presence of Cytodex augmented the stimulation significantly at 'high' cell densities but did not affect, or decrease, the stimulation slightly at 'low' densities. The beads did not cause any augmentation of thymidine incorporation in cells cultured without Con-A. The results in Fig. 1a indicate that the bead-induced potentiation of stimulation was the same in T-enriched as in unfractionated cells. Figure 1d demonstrates that

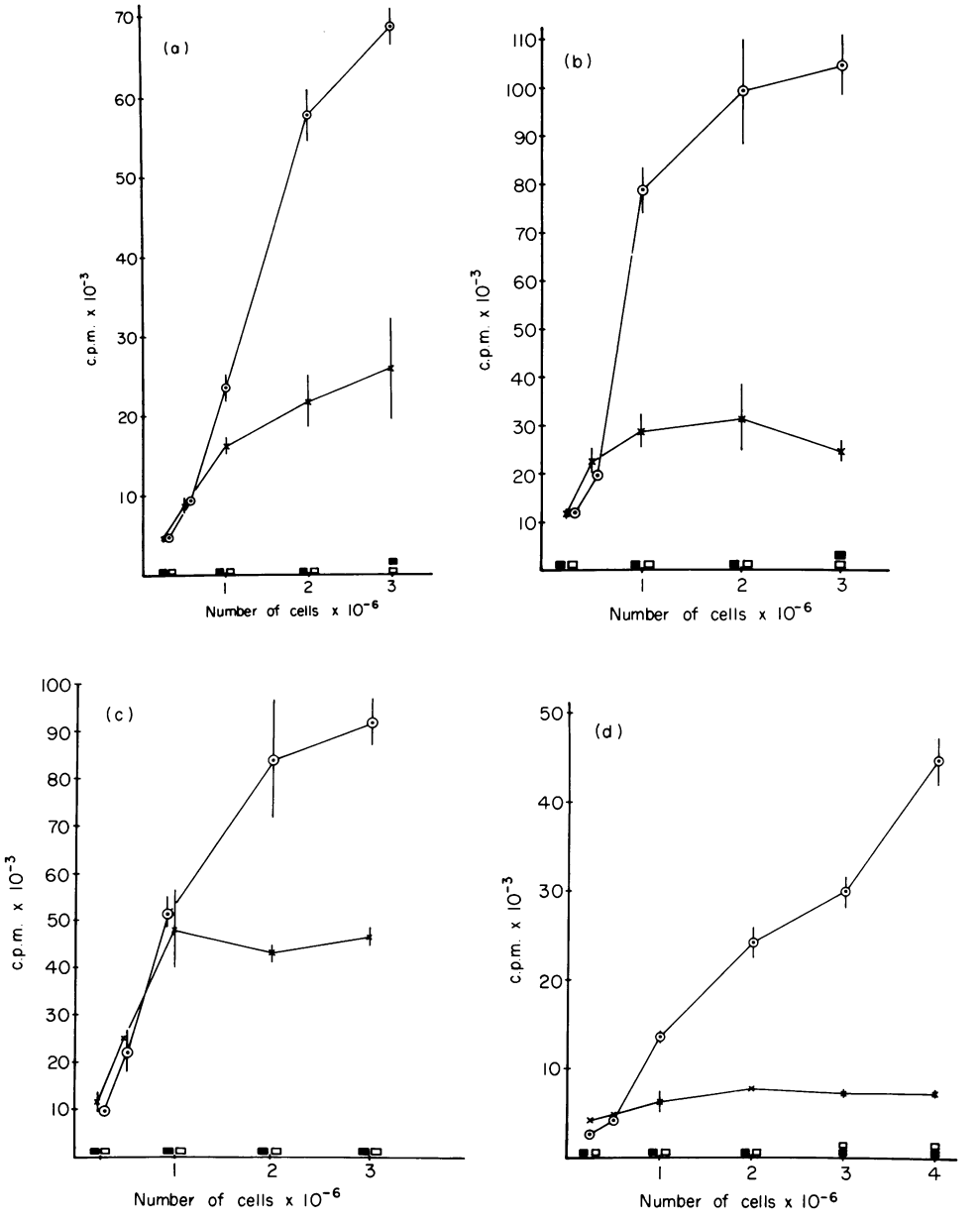

Figure 1. The influence of Cytodex cell culture beads on the stimulation of lymphocytes from four separate individuals (a, b, c and d) with Con-A 10 μg ml^{-1} in 1 ml medium. The experiment shown in Fig. 1a was performed with T-enriched lymphocytes. (× —— ×) Cells stimulated with Con-A alone; (o——o) cells stimulated with Con-A in the presence of Cytodex; (■——■) cells kept in medium only; (□——□) cells kept in medium in the presence of Cytodex. Each value is the mean of triplicate cultures. The vertical bars represent standard deviation.

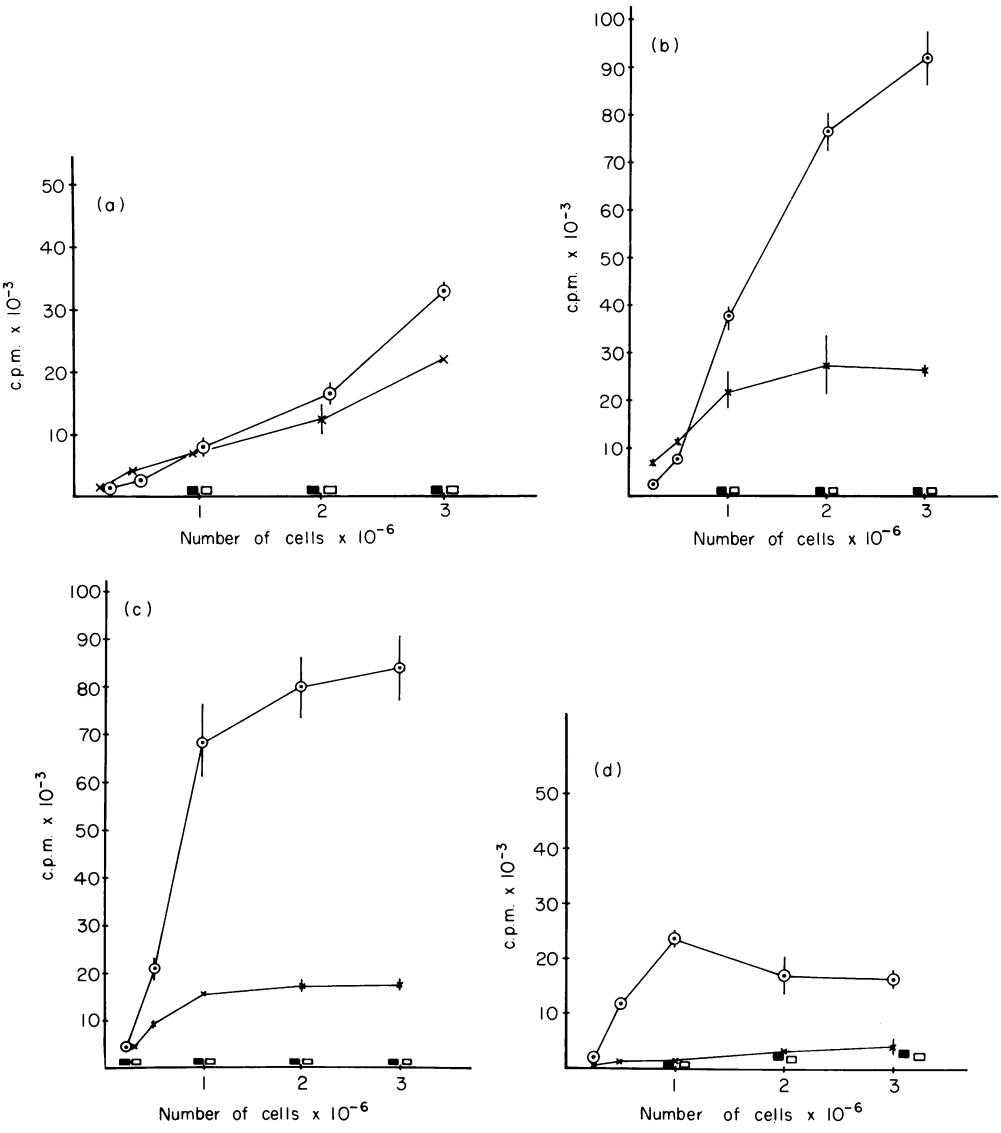

Figure 2. The influence of Cytodex cell culture beads on lymphocyte stimulation in relation to the length of the culture period. The cells were stimulated by Con-A 10 μg ml^{-1} in 1 ml medium for 40 (a), 70 (b), 90 (c) and 140 (d) hr respectively. The symbols are identical to those in Fig. 1. Each value is the mean of triplicate cultures.

some individuals exhibited very little stimulation in the absence of beads and here the bead-induced potentiating effect was dramatic. Figure 2a,b,c,d contains data concerning the effect of beads on lymphocyte stimulation at different times during a 6-day cultivation period. It can be seen that the bead-induced augmentation of the stimulation was relatively small on day 2 and pronounced on day 3, 4 and 6.

Thus, the presence of beads rendered DNA synthesis proportional to or nearly proportional to the number of cells in the cultures also at the high cell densities where such a proportionality did not exist in the absence of beads. The potentiating effect of beads

on the stimulation seemed to increase in relation to the length of the cultivation period.

The experiment shown in Fig. 3 is a comparison of Con-A-induced stimulation of varying numbers of mononuclear cells in the same medium volume in round-bottomed tubes and flat-bottomed petri dishes, respectively, in the presence and absence of Cytodex. In the tubes the incorporation of thymidine was much lower than expected at high densities and here the beads exerted a significant potentiating effect in agreement with the results in Fig. 1. In the petri dishes the incorporation of thymidine was proportional to the number of cells in the cultures at all cell densities employed both in the presence and absence of beads. The incorporation of thymidine in the petri dish cultures in the presence of beads was, however, consistently somewhat lower than in the absence of beads under the same conditions.

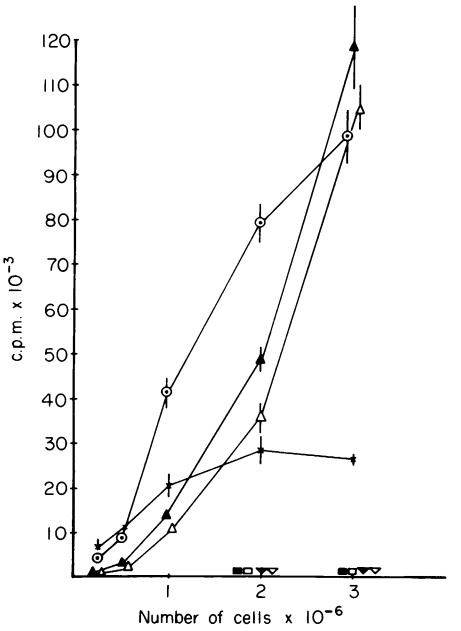

Figure 3. The influence of Cytodex cell culture beads on lymphocyte stimulation by Con-A 10 μg ml^{-1} in flat-bottomed petri dishes (Falcon 3001, diameter 35 mm) and round-bottomed tubes (diameter 10 mm) in 2 ml medium. The symbols denote: cell cultures with Con-A in tubes in the absence (×——×) and presence (○——○) of Cytodex; cultures with Con-A in petri dishes in the absence (▲——▲) and presence (△——△) of Cytodex; cultures in tubes in medium alone in the absence (■——■) and presence (□——□) of Cytodex; cultures in petri dishes in medium alone in the absence (▼——▼) and presence (▽——▽) of Cytodex.

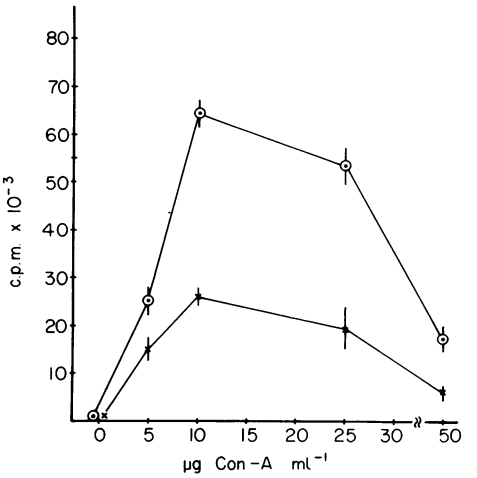

Figure 4. Dose–response curve showing the incorporation of [^{14}C]-thymidine after stimulation by different concentrations of Con-A in the absence and presence of Cytodex in 1 ml medium. The symbols are the same as in Figs 1 and 2. Each value is the mean of triplicate cultures.

Thus, the magnitude of the stimulation in tubes was essentially the same as that in petri dishes provided Cytodex was added to the tubes. It follows from this result that the potentiating effect of Cytodex is not a general one but strongly dependent on the type of culture vessel used. It is obvious that Cytodex is non-mitogenic from the fact that lymphocytes kept in medium with Cytodex in the absence of Con-A exhibited approximately the same level of thymidine incorporation as medium controls without Con-A. Additional proof that Cytodex is non-mitogenic is provided by the lack of potentiating effect by beads in the flat-bottomed culture vessels in the experiment shown in Fig 3. It can be seen in the dose–response curve in Fig. 4 that the presence of Cytodex does not change the principal relationship between Con-A concentration and mitogen response.

Visualization of the interaction between lymphocytes and substratum

Figure 5a and b illustrate the interaction between Cytodex and lymphocytes. The cells attached to the positively-charged beads both in the presence (Fig. 5a) and absence (Fig. 5b) of Con-A. The number of bead-attached cells was related to the cell density in the cultures being most pronounced at high density. In the

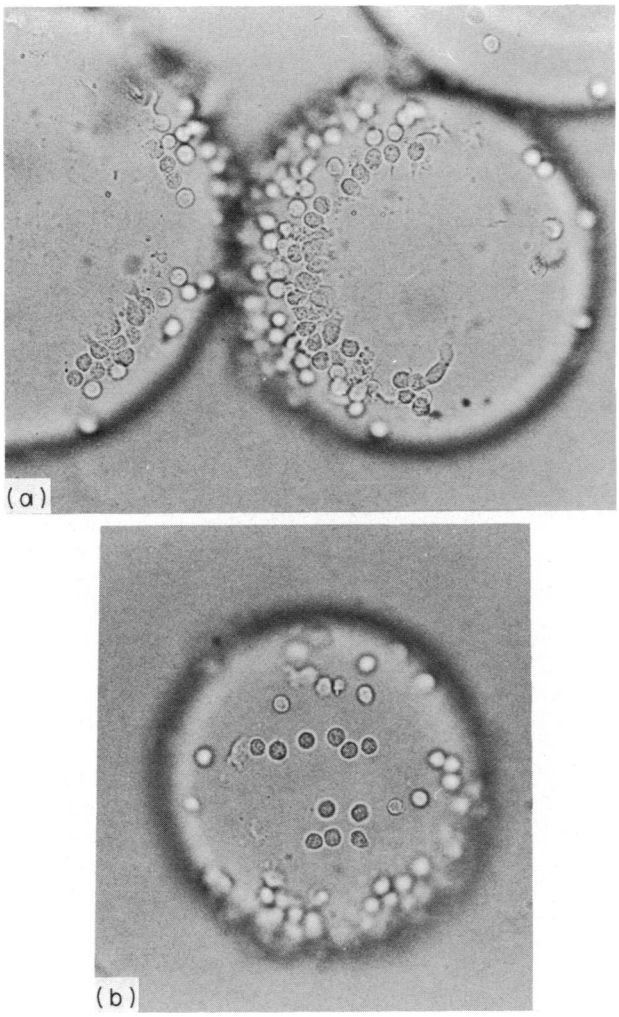

Figure 5. Bead-attached T-enriched lymphocytes at a density of 10^6 cells ml^{-1} cultured overnight in the presence (a) and absence (b) of Con-A 10 μg ml^{-1}.

Table 1. Stimulation of lymphocytes on adhesive and non-adhesive plastic

	Response c.p.m./culture†					
	Cultures on bacterial plastic			Cultures on tissue culture plastic		
Exp.	Medium	Con-A 10 μg ml^{-1}	Con-A 25 μg ml^{-1}	Medium	Con-A 10 μg ml^{-1}	Con-A 25 μg ml^{-1}
1	112±9	27,965±1089	—	103±20	23,412±2608	—
2	606±43	59,401±1401	58,840±4093	617±152	60,471±6849	45,995±5615
3*	205±37	125,468±5269	52,880±9759	185±22	186,077±9599	74,612±4300
4	128±11	52,752±2819	—	191±21	56,907±4914	—
5	426±234	36,698±2805	21,556±3179	425±179	28,598±2427	14,431±2815

* Experiment 3 was performed with 2×10^6 cells per culture and experiments 1, 2, 4 and 5 with 1×10^6 cells per culture.

† Mean of triplicate tubes ±standard deviation.

presence of Con-A 10μg ml^{-1} virtually all bead-attached cells flattened and many appeared to be motile. As a consequence of this active behaviour the cells accumulated in colonies. In contrast, the bead-attached cells in the absence of Con-A remained spherical and did not move together into colonies.

Lymphocyte stimulation and substratum adhesiveness

Table 1 is a comparison of Con-A-induced lymphocyte stimulation in adhesive tissue-culture plastic petri dishes (Falcon 3001) and non-adhesive bacterial plastic petri dishes (Falcon 1008). In most of the individuals tested there was no significant difference between the magnitude of lymphocyte stimulation in these two petri dishes. With cells from one individual there was a depression of the stimulation on bacterial plastic when compared with the results on tissue culture plastic. The cells from another individual, however, exhibited a slightly higher thymidine incorporation on bacterial plastic than on tissue culture plastic.

DISCUSSION

The major observations in the present study may be summarized as follows.

(1) *High cell density inhibited Con-A-induced DNA synthesis in unfractionated and T-enriched lymphocytes.*

(2) *Interaction of the cells with a non-cellular surface abolished the cell density-dependent inhibition of DNA synthesis. Furthermore, the interaction with the solid surface of the bead lead to proportionality between the magnitude of the mitogen response and the number of stimulated cells.* Another approach that yielded proportionality between DNA synthesis and cell number was the use of culture vessels with a larger bottom area. Under such conditions the presence of beads did not augment DNA synthesis.

These data provide unequivocal evidence that substrate contact can exert a marked potentiating effect on lymphocyte stimulation. A prerequisite for this potentiation seems to be that the availability of adhesive surface is a limiting factor. The presence of beads did not change the relationship between mitogen response and Con-A dose and the beads were not mitogenic in the absence of Con-A. In addition, the beads did not augment but rather depressed the stimulation in petri dishes. These results strongly indicate that the augmentation was a result of the anchorage of cells to beads as illustrated in Fig. 5 and not due to an increased mitogenic effect of Con-A bound to Cytodex.

Greaves & Bauminger (1972) found that B lymphocytes could be activated by Con-A covalently coupled to Sepharose beads. It is probably irrelevant to compare their data directly with ours since they used mouse lymphocytes whereas we have used human cells and soluble mitogen.

We will consider two interpretations of these findings. The first one comprises the possible function of the beads as a substratum for cell spreading. Accordingly, one explanation for the potentiating effect of beads on the stimulation is that they improve the function of an anchorage-dependent accessory cell, for example macrophages. Alternatively, the bead-induced augmentation is related to the fact that Con-A and phytohaemagglutinin (PHA) induce spreading of normally non-adherent lymphocytes (Wanger & Sundqvist, 1980; Sundqvist & Wanger, 1980). It is of interest in this context that a tight coupling seems to exist between the degree of spreading and DNA synthesis in anchorage-dependent cells (Folkman & Moscona, 1978). Consequently, the fact that growth-inducing molecules as Con-A and PHA provoke spreading of lymphocytes may reflect a role for spreading in the activation of these cells. However, because the magnitude of thymidine incorporation on adhesive and non-adhesive plastic was essentially the same it follows that lymphocyte stimulation seems to be independent of spreading. This argues against the possibility that the mechanism of action of the beads is to provide solid matrix for an anchorage-dependent cell type. However, it is not impossible that merely cell-surface contact with substratum may give rise to regulatory responses in lymphocytes or accessory cells. Compatible with this idea is the recent report that contact with a surface under conditions where subsequent spreading was prevented restored protein and RNA synthesis in anchorage-dependent fibroblasts grown in suspension culture (Ben-Zéev, Farmer & Penman, 1980).

A different rather plausible interpretation of the bead-induced augmentation of stimulation at high cell density is that the presence of beads influences cell–cell interaction. The probability of cell–cell contact is greatest in high density cultures. We postulate that some cell–cell interactions, at high cell density, probably between aggregated cells, are growth-inhibitory. The attachment of cells to beads counteracts agglu-

tination and may therefore be expected to prevent such inhibitory interactions. This hypothetical density-dependent inhibition between aggregated cells may be attributed to a specific membrane associated signal mechanism or to impaired nutrition. However, it is obvious from the illustrations in Fig. 5 of T-enriched lymphocytes in monolayer on the surface of a bead in the presence of Con-A that there was close contact also between the bead-attached cells. This means that the beads did not inhibit cell–cell interaction but modified the nature of this interaction. The evidence that the mitogen markedly enhanced the interaction between cells in monolayer on the beads prompts further investigation.

The concept that cell density has an important regulating influence on mitogen-induced lymphocyte activation has been stressed previously by Farrant & Knight (1979). They have proposed that the helper and suppressor effect of lymphoid cells are mediated through changes in the concentrations of interacting cells rather than by distinct subpopulations of lymphocytes. The variability in density-dependent inhibition and bead-induced enhancement of lymphocyte stimulation in different individuals, as suggested by the results in Fig. 1a,b,c,d, points to the possibility that the inhibition is not merely a reflection of cell density but also a characteristic property of certain individuals. Some cell type present in large quantity in their cultures may be an efficient mediator of the density-dependent growth inhibition. The present results suggest that this inhibition may be an important regulatory mechanism in lymphocyte proliferation. It is worth noting in this context that the magnitude of the inhibition increased during the cultivation period. Further studies are necessary to elucidate the basis of the density-dependent inhibition of lymphocyte stimulation.

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