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Early biochemical events associated with lymphocyte activation in ageing

I. EVIDENCE THAT Ca^{2+} DEPENDENT PROCESSES INDUCED BY PHA ARE IMPAIRED

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Summary. The requirement for Ca^{2+}, a divalent ion which plays a fundamental role in cell activation, has been analysed in cultures of PHA-stimulated human peripheral blood lymphocytes (PHA–PBL) from adult (range: 20-35 years) and old (over 70 years) subjects. For this purpose, increasing concentrations of Ca^{2+} chelators (EGTA and EDTA) were added to cultures in order to compare the effect of progressive extracellular Ca^{2+} (Ca^{2+}_{EC}) depletion on [^{3}H]-Tdr incorporation by PHA–PBL.

Kinetic analysis showed that Ca^{2+}_{EC} requirement was restricted to the first 24 h after culture initiation.

At optimal doses of PHA, the PHA–PBL from old subjects were more sensitive than those from adult subjects to increasing concentrations of both chelators. They also required larger amounts of Ca^{2+} supplements to restore their normal response after total inhibition by EGTA.

Furthermore, the PHA–PBL from the elderly were hypersensitive to verapamil (Isoptin), a drug which instigates a reversible inhibition of Ca^{2+}-dependent processes associated with lymphocyte transformation, by a quite similar action to that induced by chelators.

We conclude that the Ca^{2+}-dependent processes in lymphocyte activation are impaired with ageing.

Following further experiments and recent work suggesting that lymphocytes need more than one signal to proliferate, the authors speculate on a deficiency of a late activation signal requiring cell–cell interactions in the elderly.

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INTRODUCTION

The DNA synthesis of human blood lymphocytes stimulated by phytohaemagglutinin (PHA) decreases with ageing (Pisciotta, Wesring, Deprey & Walsh, 1967; Hallgren, Buckley, Gilbertson & Yunis, 1973; Delespesse, Duchateau, Bastenie, Lauvaux, Collet & Govaerts, 1974; Kishimoto, Tomino, Inomata, Kotegawa, Saïto, Kuroki, Mitsuya & Hisamitsu, 1978). This abnormality is principally attributed to an intracellular defect (Price & Makinodan, 1972a; Heidrick, 1973; Makinodan, Good & Kay, 1977). Indeed, serum factors from young or old subjects scarcely modify the lymphocyte response to the mitogens (Price & Makinodan, 1972b). Studies based on the identification of blood lymphocyte subpopulations show only a slight decrease of T-cell numbers that does not seem sufficient to account for the depressed immunological functions (Del Pozo-Perez, Prieto-Valtueno, Gonza-

lez-Guilabert & Velasco-Alfonso, 1973; Augener, Cohen, Reuter & Brittinger, 1974; Carosella, Mochanko & Braun, 1974; Diaz-Jouanen, Williams & Strickland, 1975; Steel, 1975; Alexopoulos & Babitis, 1976).

The reduction of DNA synthesis is more marked after 48 and 72 h of culture (optimal delay for PHA-induced DNA synthesis) (Inkeles, Innes, Kuntz, Kadish & Weksler, 1977). According to kinetic studies on the lymphocyte activation processes, it may be assumed that most cells are then in the S phase of first mitosis (Jasinska, Steffen & Michalowski, 1970; Sören, 1973).

The number and the affinity of the PHA receptors on the lymphocyte membrane are unaffected with ageing (Hung, Perkins & Yang, 1975). Consequently, the abnormalities inducing the functional defect must occur during the membrane and metabolic events of G0–G1 transition, G1 and S phases of mitosis.

Numerous studies give convincing evidence that Ca^{2+} plays a fundamental role in cellular activation, Ca^{2+} depletion in the culture medium prevents lymphocyte activation by mitogens (Alford, 1970; Whitney & Sutherland, 1972; Diamantstein & Ulmer, 1975). Furthermore, Ca^{2+} uptake is associated with the processes which transmit the first signal of lymphocyte activation (Alwood, Asherson, Davey & Goodford, 1971; Whitney & Sutherland, 1973a; Freedman, Raff & Gomperts, 1975).

The aim of this work was to compare the Ca^{2+} requirments of PHA-activated peripheral blood lymphocytes (PHA–PBL) between PHA–PBL from old subjects (PHA–PBL O) and PHA–PBL from adult subjects (PHA–PBL A) by modulating the Ca^{2+} concentrations of the culture medium with divalent cation chelators (EGTA and EDTA). The sensitivity of PHA–PBL O and PHA–PBL A to verapamil (an organic Ca^{2+} antagonist) has also been investigated (Fleckenstein, 1970).

MATERIALS AND METHODS

Subjects

Blood was collected from twenty old (age: 81·4 ± 5·8 years ± SEM, twelve females and eight males) and twenty adult (age: 27·4 ± 8·2 years ± SEM, ten females and ten males) subjects. All of them were healthy and untreated.

Reagents

EDTA: Ethylene diaminetetraacetate-sodium (Merck As.); EGTA: Ethylenglycol-bis (β aminoethylether)-NN′-tetraacetic acid, (Sigma); verapamil: Isoptin (α-isopropyl-α-(N-methyl-N-homoveratryl)-j-aminopropyl-3,4-dimethoxyphenylacetonitril-hydrochloride) (Knoll AG); Phytohaemagglutinin-P (PHA) (Wellcome); [^{3}H]-thymidine: [^{3}H]-Tdr, (IRE Fleurus, Belgium).

Preparation of lymphocytes

Peripheral venous blood was drawn in syringes containing calparine. After 1 h incubation at 37°, the mononuclear cells were separated by centrifugation over Histopaque 1077 (Sigma), washed three times in Ca^{2+} and Mg^{2+} free Hanks's (Flow Laboratories), and were immediately used for cell cultures. The mononuclear cell suspensions from the two groups of subjects contained approximately 70% E-rosette forming cells and 20% Ig-bearing cells.

Cell cultures

The procedure was adapted from that described earlier (Delespesse, Vryens, de Maubeuge, Kennes & Govaerts, 1977). The various lymphocyte preparations adjusted to a final concentration of 10^6 cells/ml in RPMI 1640 medium (Flow Laboratories) were supplemented with L-glutamine 1 mM/ml, gentamycin 3 μg/ml and 10% of pooled human AB serum collected from healthy adult subjects. The final Ca^{2+} concentration was 0·65 mM; 2×10^5 cells/well were distributed in rigid polystyrene microtitration plate FB shape (Flow Laboratories) and incubated at 37° in a 5% CO_2 incubator for 72 h. Each culture was performed in triplicate. The cultures were pulsed with [^{3}H]-thymidine (s.a. 10 c/mM, 0·5 μC/well) after 60 h and harvested on glass wool filters 12 h later with a Titertek Cell Harvester (Flow Laboratories). The filters were dried and counted for 1 min in the presence of 5 ml of liquid scintillation (Bray's solution) in a liquid scintillation counter.

The lymphocytes were cultured without PHA (control) or with suboptimal (final concentration 0·1 μg/ml) and optimal (final concentration 1 μg/ml) doses of PHA added at the start of the culture.

EDTA, EGTA and verapamil were dissolved in Ca^{2+} and Mg^{2+} free Hanks's; 100 μl of each reagent were added to 900 μl of culture medium in order to obtain the desired final concentration. The cells were preincubated at 37° for 30 min before the addition of PHA. These concentrations did not inhibit the spontaneous DNA synthesis of unstimulated PBL and

toxic effects were excluded by the trypan blue exclusion test.

Statistical analysis

Each experiment was performed in triplicate and the mean of results (coefficient of variation less than 5%) was used for statistical evaluation.

Comparisons between groups were done using two-way analysis of variance (program Packard STI-07A).

RESULTS

Comparison between maximum [^{3}H]-Tdr incorporation by PHA-stimulated PBL from old and adult subjects

Preliminary experiments (data not shown) were made to determine the optimal (PHA: 1 μg/ml) (PHA_1) and the suboptimal (PHA: 0·1 μg/ml) ($PHA_{0·1}$) concentrations of PBL from old and adult subjects. These concentrations were then used to compare the lymphocyte response to PHA in both groups. The results of these experiments showed a significant depression of optimal PHA-stimulated PBL in old subjects ($P \leqslant 0·01$) while no difference could be found at suboptimal concentrations of PHA (comments under figures).

Inhibitory effect of EGTA and EDTA on PHA–PBL from old and adult subjects

Increasing concentration of EGTA progressively lowered the [^{3}H]-Tdr incorporation induced by both concentrations of PHA (Fig. 1).

EGTA 0·4 mM inhibited the lymphocyte response from old subjects to optimal doses of PHA more than that from adult subjects ($P \leqslant 0·01$). However, at suboptimal doses of PHA, the slight difference in sensitivity to EGTA 0·4 mM between the two groups was not significant ($P \leqslant 0·05$) because of the variable EGTA effects in adults.

EDTA also lowered the PBL response to optimal and suboptimal concentrations of PHA. As was observed for EGTA at 1 μg/ml of PHA, the lymphocytes from the elderly clearly appeared to be more affected by EDTA when compared to the lymphocytes from adult subjects (0·1 mM EDTA, $P \leqslant 0·01$; 0·05 mM EDTA, $P \leqslant 0·01$; 0·25 mM EDTA, $P \leqslant 0·05$) (Fig. 1). Nevertheless, suboptimal conditions ($PHA_{0·1}$–PBL), the dose–response curves to EDTA were similar in both populations.

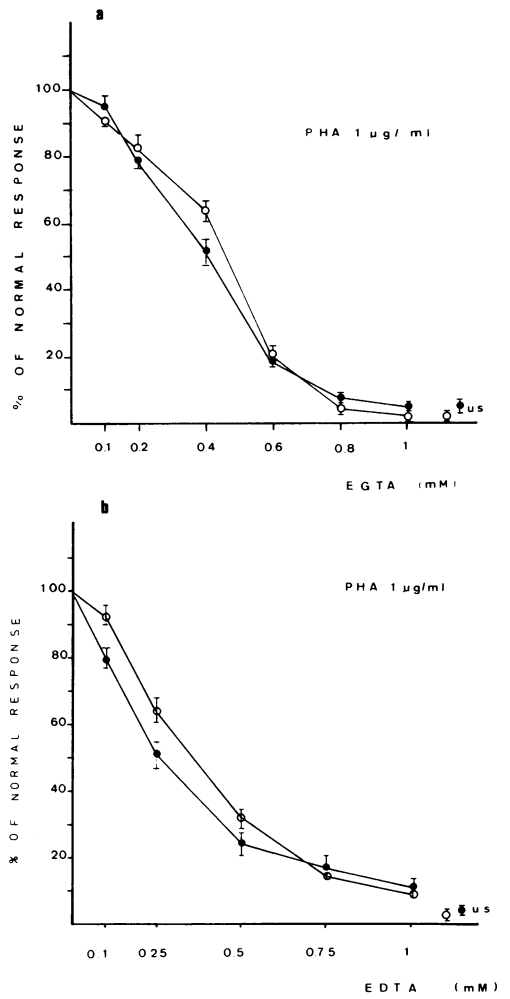

Figure 1. Action of EGTA and EDTA on [^{3}H]-Tdr incorporation of PHA-stimulated lymphocytes from adult and old subjects. The effect of EGTA or EDTA was tested as described in materials and methods. 1a. EGTA: 15 paired experiments were compared at 1 μg/ml of PHA (maximum [^{3}H]-Tdr incorp.: adult 182 ± 20 c.p.m./culture ± SEM; old: 57 ± 8 c.p.m./culture ± SEM. $P \leqslant 0·01$) 1b. EDTA: 8 paired experiments were compared at 1 μg/ml of PHA ([^{3}H]-Tdr incorp.: adult: $181 \pm 26 \times 10^3$ c.p.m./culture ± SEM; old: $85 \pm 24 \times 10^3$ c.p.m./culture ± SEM. $P \leqslant 0·05$). Each experiment was performed in triplicate and the mean value used (standard deviation less than 5%). EDTA and EGTA were added 30 min prior to PHA. Results are expressed as percentage of maximum response to PHA obtained in the absence of the chelators; •, old; ○, adult; u.s., thymidine incorporation of unstimulated cells in percentage of maximum response to PHA.

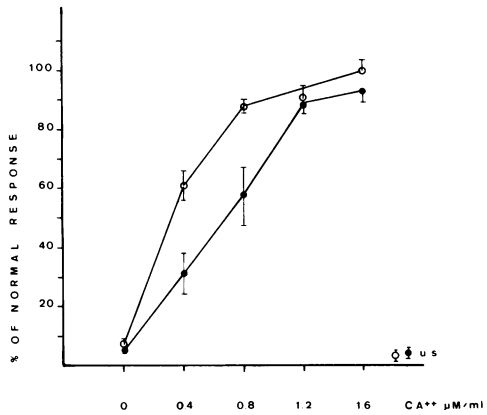

Figure 2. Reversibility induced by the addition of Ca^{2+} to the culture medium of the EGTA (1 mM) inhibitory effect on PHA-stimulated lymphocytes from adult (o) and old (•) subjects. Four paired experiments were compared at 1 μg/ml of PHA ([^{3}H]-Tdr incorp.: adult: $202 \pm 21 \times 10^3$ c.p.m./culture ± SEM; old: $82 \pm 17 \times 10^3$ c.p.m./culture ± SEM, $P \leqslant 0{\cdot}01$). The lymphocytes were preincubated for 30 for 30 min with EGTA 1 mM before PHA addition and Ca^{2+} supplements. For u.s. and expression of results see Fig. 1.

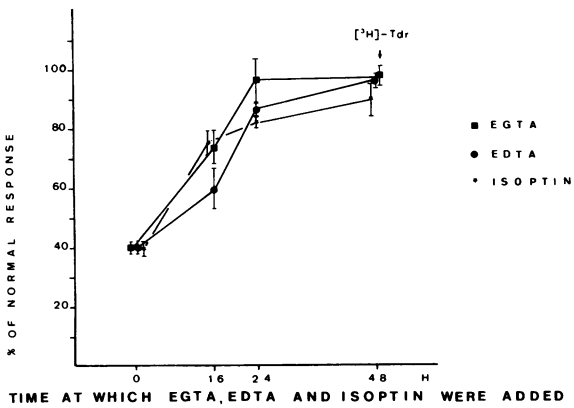

Figure 3. The kinetic of the inhibitory actions of EGTA 0·4mM, EDTA 0·5mM and verapamil on the PHA (1 μg/ml) induced stimulation of human lymphocytes from four adult subjects (age: 28 ± 3 years; maximal response $95 \pm 2 \times 10^3$ c.p.m./culture ± SEM). The reagents were added 30 min prior to PHA or at the indicated times after the addition of the mitogen. The cultures were pulsed with 0·5 μC of [^{3}H]-Tdr after 48 h and stopped 24 h later. Each experiment was performed in triplicate and the mean values used (standard deviation less than 5%). Results are expressed in percentage of maximum [^{3}H]-Tdr incorporation obtained with PHA alone.

Effect of Ca^{2+} supplements on the inhibitory action of chelators

Supplements of Ca^{2+} totally counteracted the inhibition induced by EGTA in control experiments. However the EDTA effect could not be reversed completely by adding Ca^{2+} (data not shown). The Ca^{2+} amounts required to restore a normal response to PHA in the presence of EGTA 1 mM were then compared in lymphocyte cultures from old and adult subjects. Evidence was found that stimulated lymphocytes from old needed larger amounts of Ca^{2+} than those from adult subjects ($P \leqslant 0{\cdot}05$ for Ca^{2+} 0·4 μM/ml and Ca^{2+} 0·8 μM/ml) (Fig. 2).

Kinetics of the EGTA and EDTA actions

In order to define the period during which the cells are sensitive to EDTA and EGTA, the chelators were introduced into the culture 30 min prior to or at various times after the addition of PHA (1 μg/ml) (Fig. 3).

More than 50% of the inhibitory effect was lost when chelators were added 16 h after the initiation of the culture. Both agents did not affect the [^{3}H]-Tdr incorporation if introduced simultaneously with the thymidine and their kinetics were comparable.

Inhibitory effect of verapamil on PHA-PBL from adult and old subjects

Verapamil lowered the [^{3}H]-Tdr incorporation of both optimally and suboptimally PHA-stimulated cells for concentrations inactive on unstimulated (Fig. 4). The maximum inhibition was observed with poorly stimulated cells.

The kinetics of verapamil action seemed to be comparable to the kinetics of chelators (Fig. 3). However, a slight effect on the [^{3}H]-Tdr incorporation could be suspected when both verapamil (30 μg/ml) and thymidine were simultaneously added after 48 h of culture. Furthermore, the verapamil induced inhibition of PHA–PBL was counteracted by increasing $Ca^{2+}{}_{EC}$ concentrations in culture medium (data not shown). As with EGTA and EDTA at optimal concentration of PHA, the lymphocytes from old subjects were more sensitive to increasing concentrations of verapamil than those from adult subjects ($P \leqslant 0{\cdot}01$) for 10, 20 and 30 μg/ml of verapamil whereas the lymphocytes stimulated by 0·1 μg/ml of PHA showed the same inhibitory dose–response curves in both groups of subjects.

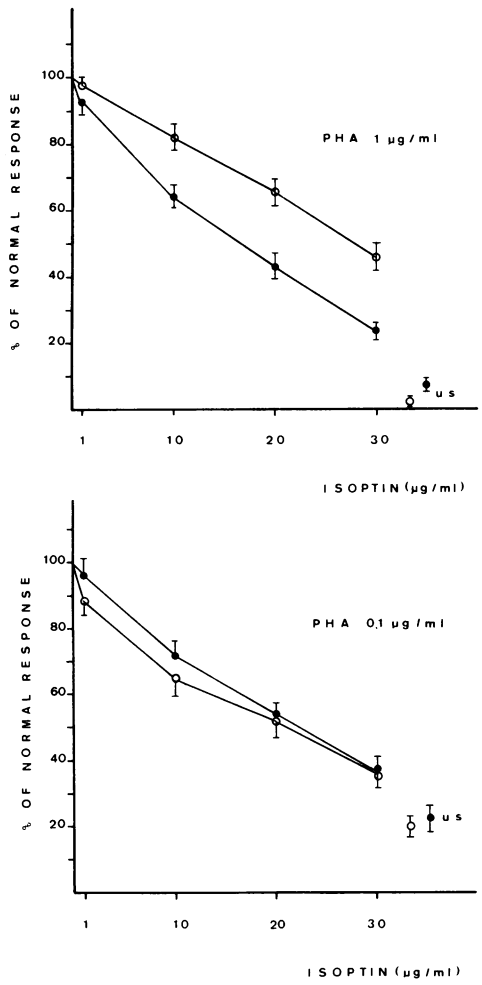

Figure 4. Action of verapamil on [^{3}H]-Tdr incorporation of PHA-stimulated lymphocytes from adult and old subjects. The lymphocytes were preincubated for 30 min at 37° before the addition of PHA. Eight paired experiments were compared at 0·1 µg/ml of PHA ([^{3}H]-Tdr incorp.: adult: $20 \pm 3 \times 10^3$ c.p.m./culture ± SEM; old: $16 \pm 3 \times 10^3$ c.p.m./culture ± SEM, n.s.) and ten paired experiments at 1 µg/ml of PHA ([^{3}H]-Tdr incorp.: adult: $179 + 22 \times 10^3$ c.p.m./culture ± SEM; old : $49 \pm 10 \times 10^3$ c.p.m./culture ± SEM, $P \leqslant 0{\cdot}01$). Each experiment was performed in triplicate and the mean value used (standard deviation less than 5%).

Results are expressed in percentage of maximum response to PHA obtained in the absence of verapamil; •, old; ○, adult; u.s., thymidine incorporation of unstimulated cells in percentage of maximum response to PHA.

DISCUSSION

There is conclusive evidence that immune functions decrease with ageing. In man, T-cell properties such as response to T mitogens (Pisciotta *et al.*, 1967; Hallgren *et al.*, 1973; Delespesse *et al.*, 1974; Kishimoto *et al.*, 1978), rejection of transplantable tumours (Goodman & Makinodan, 1975; Kishimoto *et al.*, 1978), mixed lymphocyte reactions (Alder, Takiguchi & Smith, 1971; Konen, Smith & Walford, 1973; Weksler & Hütteroth, 1974; Kishimoto *et al.*, 1978), cell-mediated cytotoxicity (Goodman & Makinodan, 1975; Kishimoto *et al.*, 1978) are more depressed than B-cell functions. The T-cell deficiency in the elderly seems to be due to an alteration in the cells and results from the inability of the immune system to generate functional T cells (Makinodan *et al.*, 1977).

It has already been stressed that some of the fundamental processes of lymphocyte activation depend on calcium (Whitney & Sutherland, 1973b; Wedner & Parker, 1976). Consequently, the analysis of Ca^{2+} requirements associated with lymphocyte transformation will provide interesting information on the early biochemical events occurring during mitogenic activation in the elderly.

EGTA, a chelator of divalent ions with a high affinity for Ca^{2+} (Caldwell, 1970) inhibited the [^{3}H]-Tdr incorporation of human PBL stimulated by optimal and suboptimal doses of PHA. These observations are in agreement with those published by others in humans and animals (Alford, 1970; Whitney & Sutherland, 1972; Diamantstein & Ulmer, 1975). The addition of equimolar amounts of Ca^{2+} to the EGTA-containing medium completely restored the lymphocyte response. This fact strongly suggests that the EGTA inhibitory effect was exclusively mediated by Ca^{2+} depletion in the culture medium.

EDTA, another chelator of divalent ions but less specific for Ca^{2+}, also inhibited the activation of PHA-stimulated lymphocytes. However, since the EDTA 0·5 mM action could not be reversed completely by adding Ca^{2+} excess, some additional effects different from $Ca^{2+}{}_{EC}$ depletion have to be suspected, as has already been reported elsewhere (Whitney & Sutherland, 1972).

EDTA and EGTA affected the lymphocyte response only during the first hours of culture. When added 16 h after the initiation of a 72 h culture, the chelators already lost 50% of their action and became inactive if added after 24 h. Furthermore, EGTA and EDTA did not influence DNA synthesis when both chelators and DNA precursors were simultaneously added after 48 h culture.

It has been shown that treatment of lymphocytes by

mitogens results in a very early increase of Ca^{2+} uptake maintained for about 30 min (Freedman, 1979). After this time, the Ca^{2+} uptake reaches that of unstimulated cells. That event is actually assumed to operate as a second messenger transmitting the signal of activation (G0 → G1 phases) through the membrane (Freedman *et al.*, 1975). The early increase of Ca^{2+} uptake results in the opening of transient Ca^{2+} channels through the plasma membrane which permit the entry of Ca^{2+}_{EC} by passive diffusion (Freedman, 1979). Thus, the depletion of Ca^{2+}_{EC} induced by EDTA and EGTA decreases the passive entry of Ca^{2+} into the cells and will obviously affect the intracellular Ca^{2+} (Ca^{2+}_{EC}) dependent events that may be located at different phases of mitosis. However, Ca^{2+}_{EC} is needed more than 30 min after the culture initiation. Therefore, it has to be suggested either that Ca^{2+}_{EC} depletion also affects cellular Ca^{2+} efflux, or that other events occurring during the first 24 h of mitosis require Ca^{2+}_{EC}.

EDTA or EGTA concentrations that suppressed the cell activation by mitogens did not affect the [^{3}H]-Tdr incorporation of unstimulated cells (data not shown). That fact strongly indicates that the Ca^{2+} requirements are principally linked to stimulated lymphocytes.

Furthermore, Whitney & Sutherland (1973b) did not find any alteration of the ^{125}I-labelled PHA fixation on specific receptors in the presence of inhibitory concentrations of Ca^{2+} chelators.

Our results demonstrate that PHA–PBL transformation requires a higher $Ca^{2+}_{EC}/Ca^{2+}_{IC}$ gradient in old than in adult subjects at optimal concentrations of the mitogen. The increased requirement of Ca^{2+}_{EC} by lymphocytes from old subjects supports the view of an impairment of Ca^{2+}-dependent mechanisms associated with cell activation in the elderly.

In order to specify some of these processes, the effect of verapamil has been tested on PBL from old and adult subjects. In PHA–PBL cultures, verapamil inhibited, by a dose-related effect, the [^{3}H]-Tdr incorporation of lymphocytes at concentrations inactive on unstimulated cells. These results are in agreement with previous studies demonstrating an inhibitory effect of the drug in concanavalin-stimulated spleen cell cultures (Blitstein-Willinger & Diamantstein, 1978).

The verapamil-inhibition was more pronounced on poorly stimulated lymphocytes. Moreover, Kinetic analysis of verapamil action was similar to those observed with chelators. Furthermore, Ca^{2+} supplements into the medium could reverse the inhibition induced by verapamil.

The foregoing supports the evidence that the drug acts on stimulated lymphocytes by antagonizing some mechanisms of Ca^{2+} uptake associated with cellular activation.

Comparison of the dose-related inhibition of the lymphocyte response between old and adult subjects denoted a higher sensitivity in the elderly. The difference was only true at optimal levels of mitogens. These last observations give very interesting information on the depressed lymphocyte response to T mitogens in relation to ageing. Indeed, results of Blitstein-Willinger & Diamantstein (1978) indicate that some processes of activation occur in the presence of verapamil, and that the cells are arrested at a predetermined stage of mitosis awaiting a late signal delivered by the mitogen.

These last observations fit in with recent work supporting the evidence that lymphocytes require more than one signal of activation to proliferate (Toyoshima, Iwata & Osawa, 1976). Furthermore, it has recently been found that a late signal indispensable for optimal transformation of lymphocytes needs the presence of extracellular Ca^{2+} to be effective (Goodwin, Bankhurst & Messner, 1977). That signal depends upon macrophage–lymphocyte interaction and acts principally after 16 h of the culture initiation.

According to the increased Ca^{2+} requirement and the hypersensitivity to verapamil of PHA_1–PBL O, and because the early Ca^{2+} uptake induced by PHA during the first 2 h of culture in the elderly is unaltered (Kennes, Hubert & Neve, manuscript in preparation), we postulate that such abnormalities lie in the deficiency of a late Ca^{2+}-dependent signal which requires cell–cell interaction.

The data are also in agreement with Goodwin & Messner (1979), who have demonstrated an increase sensitivity to prostaglandins (PGE_2) of PHA-stimulated lymphocytes in subjects older than 70 years. Indeed, PGE is assumed to be a humoral mediator of suppressor cells, effective several hours after activation (Goodwin *et al.*, 1977) and acting on cyclic nucleotide metabolism where Ca^{2+} plays a crucial role (Katz, Kierszenbaum & Waksman, 1978).

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