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ALTERATIONS OF TWO-DIMENSIONAL ELECTROPHORETIC MAPS IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES INDUCED BY CONCANAVALIN A

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Introduction

Since the discovery that plant lectins could induce blast transformation in normal lymphocytes (1), the mitogenic stimulation of these cells has been studied extensively as a model for both antigenic activation and growth control. Antigens characteristically initiate cell division in a much smaller proportion of the total lymphocyte population than do the non-specific mitogens (2); however, the kinetics of the response are believed to be the same. It seems likely that lectins bypass the requirement for antigen recognition and elicit the same cellular response as that resulting from specific immunological activation (3).

The majority of normal human peripheral blood lymphocytes are in a quiescent state either in vivo or under cell culture conditions and these resting cells synthesize only small amounts of RNA and protein. The addition of a mitogenic substance, such as concanavalin A (con A), induces a number of changes which include marked increases in RNA and protein synthesis, the initiation of DNA replication, and the active proliferations of lymphocytes (4). This transformation to lymphoblasts has most commonly been studied by thymidine incorporation into DNA (5) and to a lesser degree by the incorporation of radioactive amino acids (6).

The ability of lectins to stimulate lymphocytes to mature and divide, regardless of the antigenic specificity of the lymphocyte receptor, allows large numbers of cells to be analyzed. This affords the opportunity of studying the mechanisms whereby normal human peripheral blood lymphocytes

can be shifted into a growing state and the actual molecular changes that occur. This paper describes high-resolution separations of lymphocyte proteins and protein subunits obtained by two-dimensional polyacrylamide gel electrophoresis. The ISO-DALT system (7, 8) was used to resolve mainly those proteins with acidic or neutral isoelectric points. This system utilizes isoelectric focusing in urea in the first dimension coupled with sodium dodecyl sulfate electrophoresis in the second. The BASO-DALT system (9) is a nonequilibrium first dimension separation that resolves proteins with basic isoelectric points. Using both of these systems, we have quantitatively analyzed mitogen-induced lymphocyte proliferation. These analyses have been performed on isolated nuclei as well as whole cells.

Materials and Methods

Isolation of Lymphocytes. All experiments were performed on normal human peripheral blood lymphocytes. Blood was collected in heparinized tubes by venipuncture and centrifuged for 15 min at 500 x g to obtain buffy coats. Buffy coat leukocytes were diluted 1:2 in Ringer Locke's solution (0.9% NaCl, 5.4 x 10^{-3} M KCl, 0.02% $NaHCO_3$, 0.2% glucose, and 0.024% $CaCl_2$) and lymphocytes were isolated by gradient centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N. J.). Purified lymphocytes were washed in phosphate buffered saline, and residual red blood cells were lysed in 4 volumes of a 0.85% NH_4Cl solution (pH 7.4). Following erythrocyte lysis, lymphocytes were washed twice in phosphate buffered saline and resuspended in RPMI 1640 medium.

Labeling of lymphocyte proteins. Purified lymphocytes were cultured in flat-bottomed multiwell plates (Falcon, Oxnard, Calif.) at concentrations of up to 4 x 10^6 cells/ml. Cells were cultured in RPMI 1640 medium (containing no cold methionine) which was supplemented with either 10% FCS (fetal bovine serum) or the following purified serum components: human serum albumin, 6 x 10^{-6} M (Behringwerke, Marburg/Lahn, W. Germany); 1/3 iron-saturated human transferrin, 1 x 10^{-8} M (Behringwerke); soybean lipid and cholesterol, 100 µg/ml (Sigma Chem. Co., St. Louis, Mo.); and Na selenite (Na_2SiO_3,0.0173 mg/liter) as described by Iscove and Melchers (10).

Cultures also contained 4 x 10^{-5} M 2-mercaptoethanol and 25-50 μCi (^{35}S) methionine (specific activity 1200 Ci/mM, Amersham, Arlington Hts., Il.). Concanavalin A (Boehringer-Mannheim, Indianapolis, Ind.) was added at a concentration of 2.5 μg/ml to stimulate cultures. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 18 hours unless otherwise indicated.

Two-dimensional electrophoresis. At the end of the labeling period, lymphocytes were harvested and washed in phosphate buffered saline. Cell pellets were lysed in a mixture of phosphatidyl choline and urea as previously described (9). Solubilized samples were centrifuged for 1 hour at 180,000 x g in specially adapted microfuge tubes to pellet high molecular weight material such as intact DNA (11). The soluble and solubilized proteins were analyzed by high-resolution electrophoresis using the ISO-DALT system (7, 8) to resolve the acidic and neutral proteins and the BASO-DALT system (9) to resolve the basic proteins. LKB 3.5 - 10 Ampholines (LKB, Rockville, Md.) were used for ISO gels, and Serva 2-11 ampholytes (Accurate Chem. Co., Hicksville, N. Y.) were used in BASO gels. Gels were fixed, dried, and autoradiographed for 1-2 weeks as described (12).

Results and Discussion

ISO-DALT separations of the acidic and neutral proteins from human lymphocytes with and without con A stimulation demonstrated quantitative but not qualitative changes in their protein composition (Figure 1). For reference purposes, actin and intermediate filament protein are identified in all panels of Figures 1 through 4. The majority of spots showed apparent density increases after con A stimulation, with some spots increasing more than others. The two most acid spots in box 1 appear to be of nuclear origin as they are present in both nuclear and whole cell maps. On the other hand, the most basic spot indicated in the box as well as spot 4 are lost after nuclear isolation and therefore do not appear to be nuclear proteins. Spots 2 and 3 are thought to be mainly localized in the nucleus, since they are greatly enriched in panels C and D. There are also many

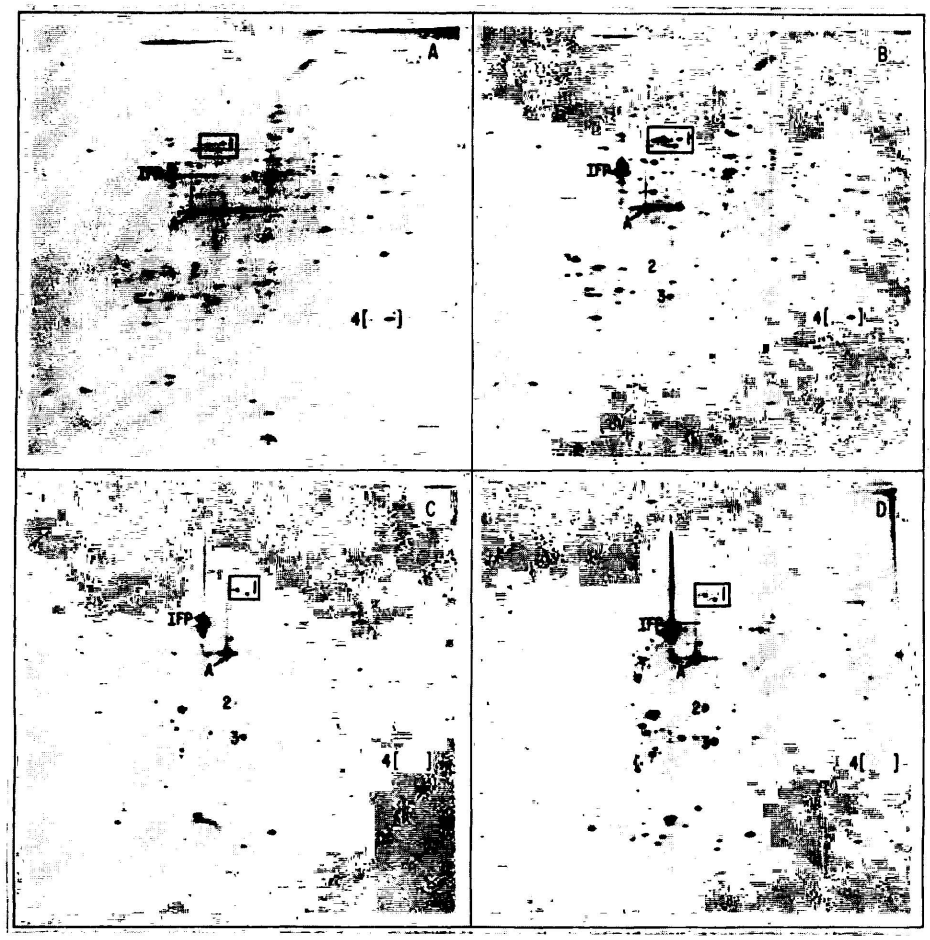

Fig. 1. ISO-DALT patterns of human peripheral blood lymphocytes from two-dimensional gel autoradiographs. The gels are oriented with the basic end to the right and the acidic end to the left. (A) unstimulated whole lymphocytes, (B) con A-stimulated lymphocytes, (C) isolated nuclei from unstimulated lymphocytes, and (D) isolated nuclei from con A-stimulated lymphocytes. Actin (A), intermediate filament protein (IFP, as identified by NLA), and spots 1-4 are labeled for reference purposes.

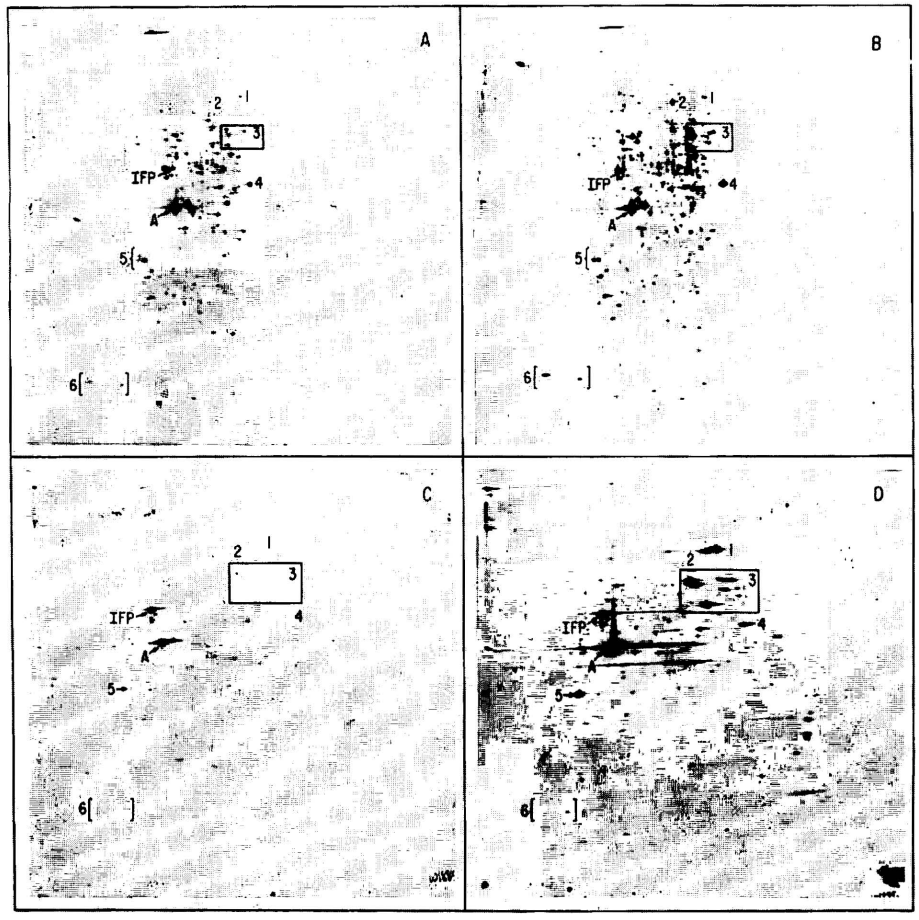

Fig. 2. BASO-DALT patterns of human peripheral blood lymphocytes from two-dimensional gel autoradiographs. The gels are oriented as in Figure 1. (A) unstimulated whole cells, (B) con A-stimulated cells, (C) isolated nuclei from unstimulated cells, and (D) isolated nuclei from con A-stimulated cells. BASO gels were electrophoresed for 2400 volt-hours. Protein identifications are the same as in Figure 1, and spots 1-6 are for reference purposes.

smaller spots (particularly the more acid, high molecular weight proteins) found in the nuclear maps that are not detectable in whole lymphocyte patterns. Most likely the increased spot densities and the appearance of new spots in the nuclei are due to a concentration effect, indicating that these proteins are present in lower concentrations relative to the whole cell, and thus are diluted out in the whole lymphocyte pattern. Alternatively, the source of some minor spots could be from degradation that occurred during the isolation procedure. The major nuclear spots appear to represent proteins that are unique to the nucleus.

The BASO-DALT procedure, which separates the more basic cellular proteins, again revealed that changes associated with con A stimulation are generally quantitative in their nature (Figure 2). Also, some spots appear to increase more than others. For example, the spots labeled 1, 3 and 4 increase more dramatically upon stimulation (Figure 2B and 2D) than the other major proteins. BASO-DALT patterns of isolated lymphocyte nuclei (Figure 2C and 2D) show increases similar to those seen in the lymphocyte pattern (Figure 2A and 2B); however, there appears to be a dramatic increase in some of the high molecular weight (spots 1 and 3) basic proteins in the stimulated nuclear patterns. Spots 2 and 6 appear to be nonnuclear proteins as they are almost completely lost after isolation of the nuclei. The three spots indicated as 5 are even more interesting, since only one is present in all four gels, while the others increase or decrease with stimulation and/or nuclear extraction. As seen in the ISO-DALT patterns (Figure 1), there were no detectable qualitative changes in the BASO-DALT patterns (Figure 2) of stimulated and unstimulated lymphocytes.

Further ISO-DALT and BASO-DALT analyses were performed at timed intervals as the lymphocytes underwent mitogen-induced blast transformation (Figures 3 and 4). De novo protein synthesis is initiated during the first hour after stimulation (results not shown) and increases rapidly during the first 18 hours of blastogenesis. In general, from 6 to 18 hours was the period of most rapid protein synthesis. However, after 18 hours, there is a general decrease in newly synthesized lymphocyte proteins and those proteins that are labeled may represent a pool of proteins synthesized at an earlier stage. Both ISO-DALT and BASO-DALT patterns of lymphocytes during

the first 24 hours after mitogen-induced blastogenesis demonstrate similar kinetics. There is a concurrent decrease in protein synthesis after 18 hours, although there are unique variations found in some of the spots.

Variation in the synthesis of individual proteins is illustrated in both Figures 3 and 4. For example, the four spots indicated as 1 (Figure 3) demonstrate interesting patterns of synthesis. Two of the spots are heavily synthesized during the entire 24-hour cycle, while the other two appear to be mainly synthesized from 6 to 18 hours. On the other hand, the four spots indicated as 2 and the doublet indicated as 3 (Figure 3) increase and decrease relative to one another. Similar variation is also seen in the BASO-DALT patterns (Figure 4). Spot 1 increases and decreases synchronously with total de novo protein synthesis. Conversely, spots 2 and 3 appear to decrease during the period of rapid protein turnover (6 to 18 hours after stimulation; Figures 4B and 4C) increasing again 18 hours after stimulation (Figure 4D). The doublet indicated as 4 has a different pattern of synthesis. The more basic spot remains constant throughout the 24-hour cycle, while the more acid spot increases and decreases relative to total protein synthesis. The histones (Figure 4) are not labeled heavily during the first 24 hours. However, in the gels stained with Coomassie Blue (not shown), they appear as major spots (equal to actin in density). This implies that while the histones in these cells (including those present prior to stimulation) are being solubilized, relatively little de novo synthesis of histones occurs during the initial stages of blast transformation. It would thus appear that in blast transformation the cell first synthesizes one set of proteins, which are indicative of blast transformation and then a second set of proteins associated with DNA replication and division.

Conclusions

These investigations demonstrate that con A-stimulated human lymphocytes are induced to undergo rapid protein synthesis. While some proteins seem to be synthesized in greater quantities than others, con A apparently does not induce any unique lymphocyte proteins during the time period examined.

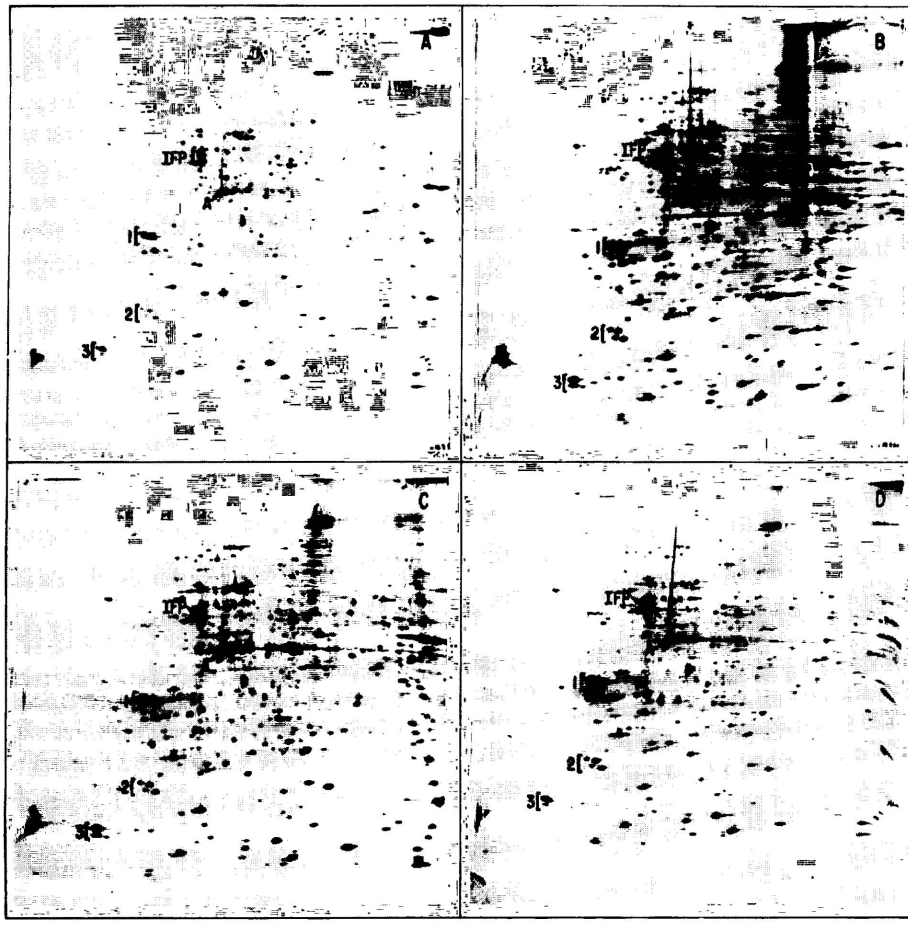

Fig. 3. Autoradiographs of ISO-DALT patterns from human peripheral blood lymphocytes induced to undergo blast transformation by con A. Lymphocytes were exposed to (^{35}S) methionine for the following time periods after initiation of the cultures: (A) 0 to 6 hours, (B) 6 to 12 hours, (C) 12 to 18 hours, and (D) 18 to 24 hours. The gels are oriented as in Figure 1. Protein identifications are the same as in Figure 1, and spots 1-3 are for reference purposes.

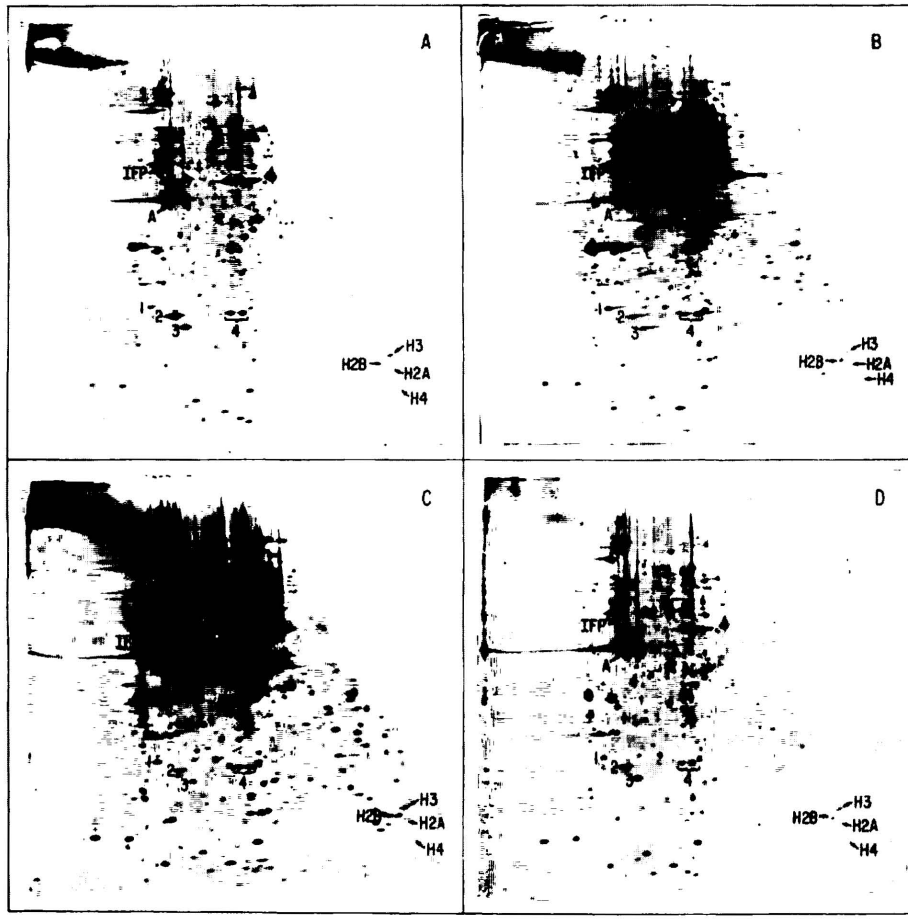

Fig. 4. Autoradiographs of BASO-DALT patterns of human peripheral blood lymphocytes induced to undergo blast transformation by con A. Lymphocytes were exposed to (^{35}S) methionine for the following time periods after initiation of the cultures: (A) 0 to 6 hours, (B) 6 to 12 hours, (C) 12 to 18 hours, and (D) 18 to 24 hours. BASO gels were electrophoresed for 2400 volt-hours. The gels are oriented as in Figure 1. Actin (A), intermediate filament protein (IFP), histone 2a (H2a), histone 2b (H2b), histone 3 (H3) and histone 4 (H4) are identified, and spots 1-4 are labeled for reference purposes.

This would substantiate the idea that mitogens induce nonspecific blast transformation in lymphocytes. The lymphocyte apparently undergoes a period of rapid protein synthesis during the first 18 hours after stimulation, followed by a decrease in de novo synthesis. This is probably an indication that the lymphocyte has shut off most cellular protein synthesis as a prelude to DNA replication and division.

Acknowledgments

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