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Two-Dimensional Analysis of Human Lymphocyte Proteins: I. An Assay for Lymphocyte Effectors

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We describe an assay for lymphocyte effectors that is capable of establishing the existence of regulators of lymphocyte gene expression (including post-transcriptional control and protein processing) and has the ability to characterize the response at the molecular level. The hypothesis that circulating effector substances excreted through the kidney can be actively present in human urine was tested with this assay. Thus, biologically active protein molecules in urine were detected at concentrations of less than 1 mg/L and over a wide range of dilutions. Activities were detected and quantitated by culturing human lymphocytes with human urinary proteins in the presence of [^{35}S]methionine and subsequently analyzing the labeled lymphocyte proteins by two-dimensional gel electrophoresis. Thus, protein analysis by two-dimensional gels was used to indirectly detect changes produced in cultured lymphocytes after exposure to regulatory molecules. Proteins or sets of lymphocyte proteins appeared or disappeared after exposure to normal or pathological human urinary proteins. Normal human urinary proteins triggered the appearance of sets of proteins referred to by number as the "Urocon" proteins and suppressed the synthesis of protein sets referred to as "Urocof" proteins. In addition to the normal alterations described, urinary proteins from individuals with influenza or acute leukemia and after renal transplantation were capable of inducing unique alterations in lymphocyte patterns.

Additional Keyphrases: *ISO-DALT system · regulation of gene expression · protein gene-products · cellular protein complement · urinary proteins · "mapping" of proteins · electrophoresis, polyacrylamide gel*

Only a few of the various regulatory molecules present in the human body are well characterized and understood. In the absence of a precise and quantitative biological assay it is difficult to isolate and characterize regulatory molecules, and to distinguish between them. The system described here, an assay for lymphocyte effectors (ALE),[1] has the potential to discover and characterize regulators of gene expression and the response that they elicit in the human lymphocyte. ALE has been used to demonstrate that human urine, desalted by use of two P-6 Biogel columns and concentrated by lyophilization, contains biologically active molecules.

Development of techniques such as the radioimmunoassay (*1*, *2*) and the enzyme-labeled immunosorbent assay (*3*, *4*) have allowed sensitive analysis for well-characterized pathological proteins. However, these assays are limited to the analysis of known effectors for which specific antibodies are available (*5*). When the target cell is capable of responding very efficiently (e.g., to low in vivo concentrations of antigens, lymphokines, or hormones), a better approach would be to use the target cell as an assay system, and to develop specific immunoassays later. Since regulatory molecules may effect their response by altering gene expression (including transcription modification, RNA processing, translation, and post-translational modification) in target cells, analysis of protein gene-products in these cells should provide a direct or indirect assay for effector molecules. Initially, using high-resolution two-dimensional electrophoresis, one can study the effects of regulators on cellular proteins without defining just where the control is exerted in the cell (*6*). In addition, we do not distinguish all of the regulatory processes involved in gene expression, but are concerned here with the end result: the appearance or disappearance of a protein in the cell.

Alterations in concentrated human urinary proteins (HUP) have been correlated with disease for several patients by use of two-dimensional electrophoresis (*7*), and we have previously demonstrated the presence of biologically active factors in normal urine (*6*). These regulatory substances were discovered by analysis of Novikoff hepatoma cells before and after treatment of the cells with HUP. The factor(s) present induced synthesis of a unique set of proteins in the hepatoma cells.

We show here that normal human urine contains biologically active substances that alter the synthesis and breakdown of specific cellular proteins in human lymphocytes. Thus, production of certain proteins or sets of proteins can be turned both on or off by these regulatory molecules. Furthermore, some pathological urine samples contain substances that differentially alter lymphocyte protein patterns. Analysis of the protein constituents in human peripheral blood lymphocytes before and after treatment with HUP from a variety of patients is therefore a sensitive differential method of assessing active substances in human urine.

Materials and Methods

Isolation of Lymphocytes

All experiments were performed on human peripheral blood lymphocytes from normal and pathological donors. Blood was collected by venipuncture into heparinized tubes and was centrifuged for 15 min at 500 × *g* to obtain buffy coats. Buffy-coat leukocytes were diluted threefold in Locke's solution (per liter: 9 g of NaCl, 5.4 mmol of KCl, 0.2 g of $NaHCO_3$, 2 g of glucose, and 2.4 mg of $CaCl_2$) and lymphocytes were isolated by gradient centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ 08854). Purified lymphocytes were washed in RPMI 1640 medium (GIBCO, Grand Island, NY 14672), and residual erythrocytes were lysed, if necessary, in four volumes of NH_4Cl solution (8.5 g/L, pH 7.4). After erythrocyte lysis, lymphocytes were washed twice in RPMI 1640 medium and resuspended in RPMI 1640 labeling medium containing no non-radiolabeled methionine.

Labeling of Lymphocyte Proteins.

Purified lymphocytes were cultured in flat-bottomed, multiwell plates at concentrations of up to 4 × 10^6 cells per

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[1] Nonstandard abbreviations used: ALE, assay for lymphocyte effectors; ISO-DALT, name given the two-dimensional polyacrylamide gel electrophoresis system used in the Molecular Anatomy Program; separation in the first dimension is on basis of charge, in the second on basis of mass; HUP, human urinary proteins of relative molecular mass >6000. Abbreviations used in Figures are identified in figure legends.

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milliliter. Cells were cultured in RPMI 1640 labeling medium supplemented with either fetal bovine serum (50 mL/L) or with the following purified serum components: human serum albumin, 6×10^{-6} mol/L (Behringwerke, Marburg/Lahn, F.R.G.); one-third iron-saturated human transferrin, 1×10^{-8} mol/L (Behringwerke); soybean lipid and cholesterol, 100 mg/L (Sigma Chemical Co., St. Louis, MO 63178); and sodium selenite (Na_2SeO_3, 17.3 μg/L) as described by Iscove and Melchers (*8*). Cultures also contained 2-mercaptoethanol (4×10^{-5} mol/L) and 25–50 μCi of [^{35}S]methionine (spec. acty. 1200 kCi/mol, Amersham, Arlington Hts., IL 60005). Cultures were incubated at 37 °C in a humidified atmosphere containing five volumes of CO_2 per 100 volumes, for 18–24 h unless otherwise indicated.

Preparation of Concentrated Urinary Proteins.

Fresh urine, 100 to 400 mL, was concentrated over two P-6 Biogel columns with lyophilization of the separated proteins represented by the peaks from each column (*7*, *9*). Concentrated and desalted urinary proteins were cultured with lymphocytes (up to 4×10^6) at concentrations of 1 g/L unless otherwise indicated.

Two-dimensional Electrophoresis

At the end of the labeling period, lymphocytes were harvested and washed in Dulbecco's phosphate-buffered saline (GIBCO). Cell pellets were lysed in a mixture of phosphatidylcholine and urea as previously described (*10*). Solubilized samples were centrifuged for 1 h at 222 000 × *g* in a Beckman LP42 rotor to sediment material of high molecular mass such as intact DNA (*9*, *10*). The solubilized proteins were analyzed by high-resolution two-dimensional electrophoresis (ISO-DALT system), to resolve the acidic and neutral proteins. The ISO gels used in the first-dimension separation contained LKB 3.5–10 Ampholines (LKB Instruments, Inc., Rockville, MD 20852). Internal isoelectric-point standards produced by carbamylation of rabbit-muscle creatine kinase (EC 2.7.3.2; Sigma Chemical Co.) were used in all ISO gels (*11*). Gels were fixed, dried, and autoradiographed for one to two weeks as described previously (*6*, *12*).

Set Naming

Most of the proteins studied here have not been previously described, named, or characterized. For organizational purposes, we classify the information obtained about individual spots on two-dimensional patterns into functionally related protein sets. Each set is referred to by a six-letter acronym, and every member of that set has a serial number, which follows the acronym, as previously discussed (*13*). The serial numbers need not be assigned to members of the set in any predetermined order, but numerical groupings can be used for further classification within the set. Thus, in this paper the set of proteins that appear after treatment with HUP is referred to as the "Urocon" set (factors in urine turn them on); those suppressed by substances in the urine are called "Urocof" proteins (factors in urine turn them off). This system is intended to provide useful as well as evocative names for members of sets of proteins whose identities and functions have not been described, as well as a scheme for classifying known and unknown spots.

Results

Modification of Lymphocyte Protein Synthesis by HUP

Two-dimensional patterns of human lymphocyte proteins show numerous changes after the cells are cultured with, per milliliter, 1 mg of HUP from a normal individual. Figure 1 depicts a two-dimensional pattern of human lymphocyte proteins in which the HUP-induced changes usually detected have been indicated. Synthesis of several proteins or sets of proteins is suppressed by added HUP, and these proteins will be referred to by number as members of the Urocof set. The spots labeled Urocof:1-4, Surface 5 (S5), and β_2-microglobulin (β_2-M) are lymphocyte surface proteins whose identification will be described elsewhere (Willard and Anderson, manuscript in preparation). Note that Urocof:3 is the haze around the two tropomyosin (Tm) spots. Many experiments have demonstrated that ^{35}S-labeled Urocof:1-4 are not present in patterns of cells treated with HUP. The syntheses of Surface 5 and β_2-microglobulin appear to be unaffected by treatment with HUP from any patient or normal individual. Synthesis of Urocof:21-24, a calcium-regulated protein group (unpublished data), is also suppressed by the presence of HUP in lymphocyte cultures.

The spots labeled "Varble:2" and ":3" (for "variable") are under a control mechanism not yet elucidated; however, their synthesis is affected by a large variety of unrelated chemicals, drugs, mitogens, hormones, and other compounds (data not shown here). Thus, the effect on Varble:2 and :3 does not appear to be related to HUP per se because the intensity of these spots is erratic from experiment to experiment. Urocon 51-54 are often detected in lymphocytes treated with HUP from patients with acute infections; however, they have also been detected after treatment with some "normal" samples. Because these spots can be induced by HUP from the same normal individual on occasions of illness, they may ultimately prove to be indicators of infection. The relative amount of these regulatory activities per milligram of HUP varies from individual to individual and with different specimens from the same individual.

Alterations in the myosin light chains (Urocof:31-32), actin, and other lymphocyte cytoskeletal proteins have been found in HUP-treated cultures. Although synthesis of the myosin light chains appears to be suppressed, fragments of actin and possibly of other structural proteins accumulate in HUP-treated cells (*14*). These "clipped" products were detected only if the protease inhibitor, phenylmethylsulfonyl fluoride, was used in the cell-solubilization buffer.

Titration of Human Urinary Proteins

Alterations in lymphocyte patterns were found to depend on the amount of normal HUP added to the cultures (Figure 2). The density of all HUP-regulated spots paralleled the amount of urinary proteins added. Synthesis of Urocof:1-4 (as reflected by spot intensity) began to decrease at a dose of 10 μg of HUP per milliliter (Figure 2*B*); but Urocof:21-24 did not appear to be affected until 100 μg per milliliter was added. HUP-induced (i.e., new) spots also appeared at a HUP dose of 100 μg per milliliter (Figure 2*C*). In addition, the regulation of lymphocyte proteins according to the dose of HUP gives helpful information about the co-regulation of spots. Spots that increase or decrease in their intensity together form good candidates for a set of co-regulated proteins, while two spots whose dose characteristics differ are likely to define different sets.

Kinetic Studies of HUP-Induced Alterations in Lymphocytes

Samples of normal urine were tested for their effectiveness in altering lymphocyte gene expression by culturing the cells in the presence of 1 mg of HUP per milliliter for various intervals. The effect of HUP is most dramatic in the first 6 h of culture (Figure 3). (The patterns showed no qualitative changes from 6 to 48 h, so, for simplicity, the patterns from 18-, 24-, and 48-h cultures are not shown.) Although there were no qualitative changes after 6 h, some quantitative differences

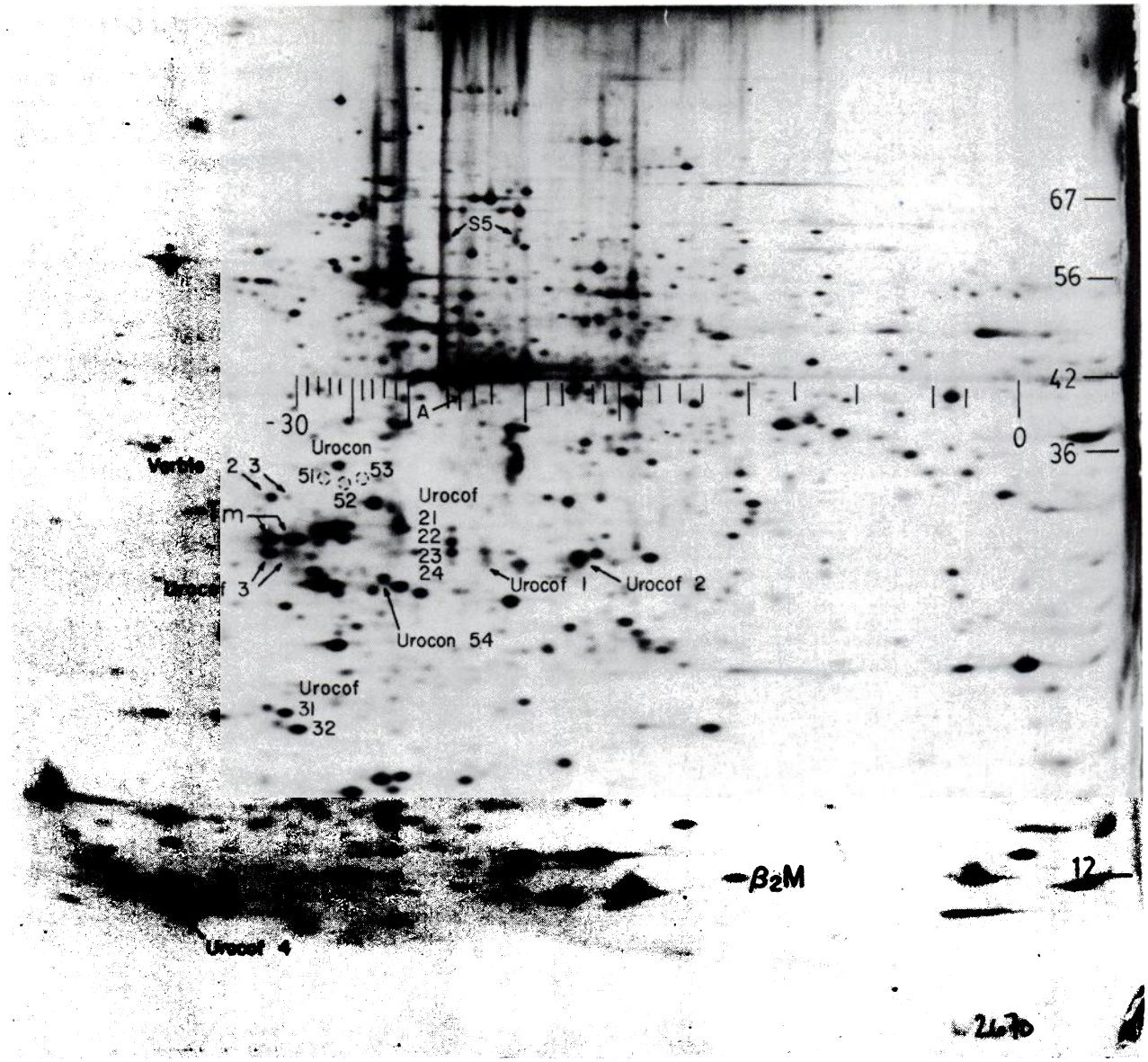

Fig. 1. Pattern of human peripheral blood lymphocytes from a two-dimensional gel autoradiograph

The cells were radiolabeled with [^{35}S]methionine for 18–24 h. The gel is oriented with the basic end to the right, acidic end to the left. ISO gels were run with LKB 3.5–10 Ampholines. Slab gels were linear gradients of 10–20% polyacrylamide from the top to the bottom of the gel. The scale running horizontally across the figure from 0 to −30 marks the position of the isoelectric point standards (*11*); the numbers along the right-hand margin indicate approximate relative molecular masses ($\times 10^{-3}$) in sodium dodecyl sulfate. Urocon and Urocof proteins are identified by number. Urocon proteins appear after culturing lymphocytes with HUP; Urocof proteins are lost after HUP treatment. Other identifications include actin (*A*), β_2-microglobulin (β_2M), non-muscle tropomyosin (*Tm*), and Surface 5 (*S5*)

(as judged by eye), not relevant to this discussion, were detectable in control and treated cultures. Several interesting patterns emerged when the effect of HUP was studied as a function of time:

(*a*) The series of spots labeled Urocof:6 is thought to be a surface glycoprotein. Appearance of this protein was delayed in HUP-treated cultures (Figure 3, *B* and *D*) over the corresponding controls (Figure 3, *A* and *C*). Labeled Urocof:6 did not appear to be present in lymphocytes after 3 h of culture (Figure 3, *G* and *H*).

(*b*) Urocof:1-4 were not heavily labeled during the first 6 h of culture in either the control or treated cells. Therefore, it is difficult to detect these spots or to discern the early effect of HUP on their synthesis.

(*c*) As for the myosin light chains (MLC, Urocof:31-32), in the case of untreated cells the amount of labeled light chains increases with time, while in the HUP-treated lymphocytes, light-chain synthesis increased during the first hour of incubation, after which labeled protein decreased until labeled myosin light chains were barely detectable at 3 h (Figure 3*H*).

(*d*) The appearance of a set of spots that we believe to be fragments of cytoskeletal proteins [Urocon 11-15; (*14*)] is rapid, with the major spots being radioactively labeled in treated cultures after 15 min of incubation (Figure 3*B*). After 6 h (Figure 3*J*) more of these fragments had accumulated, and products were also present. This set of spots was only detectable in HUP-treated cultures when the protease inhibitor phenylmethylsulfonyl fluoride was present in the lysis buffer. This group probably represents a co-regulated set of proteins, with a single regulatory molecule controlling the response.

Effect of Human Urinary Histocompatibility Antigens on Lymphocytes

We compared results of analyses performed on urinary proteins and lymphocytes from the same individual with those from different individuals. If histocompatibility antigens (HLA) were responsible for the disappearance of the Urocof

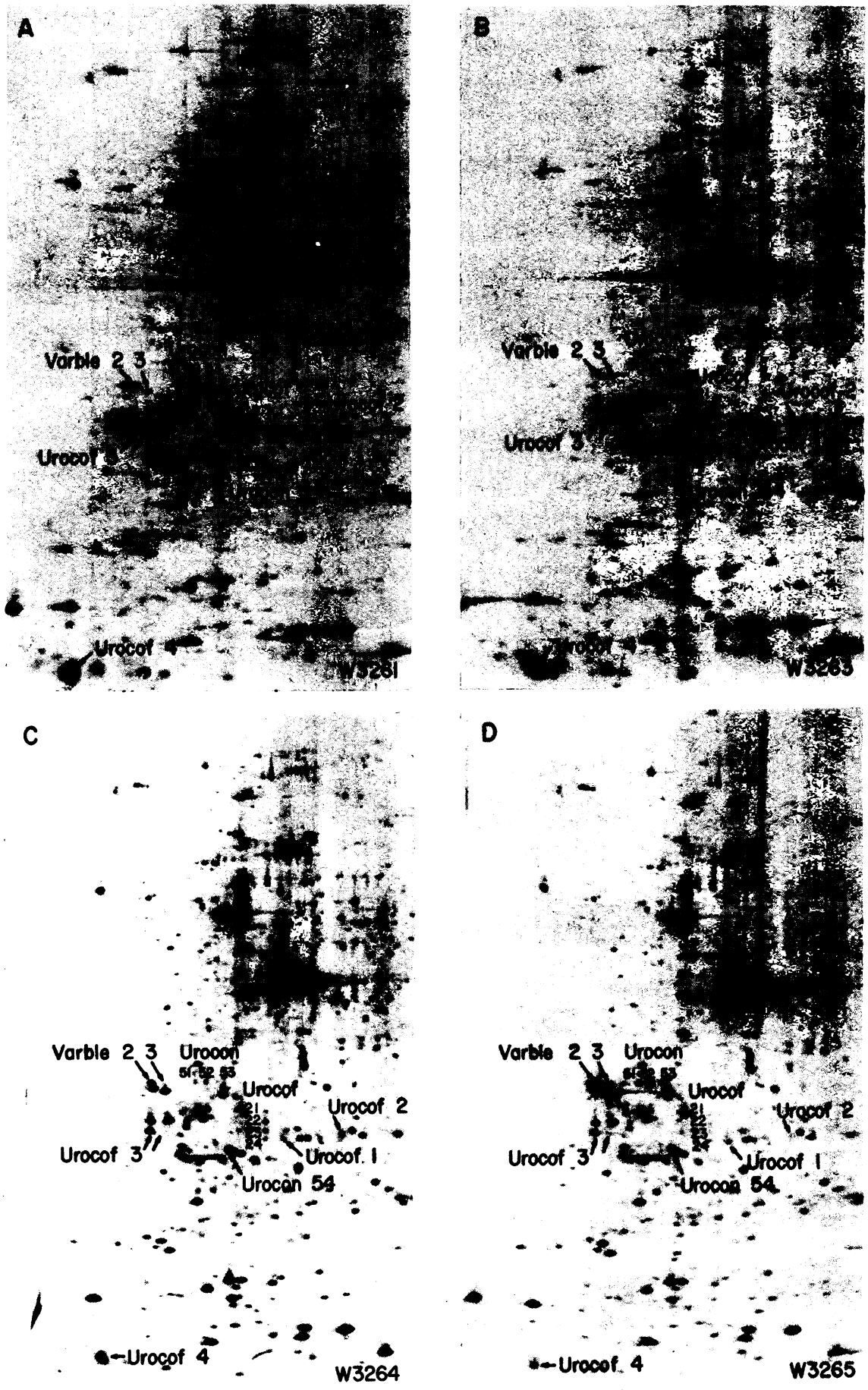

Fig. 2. Titration of the HUP effect on human peripheral blood lymphocytes

Sections from autoradiographs shown here. The cells were cultured with normal male HUP in the following concentrations: (*A*) control without urine, (*B*) 10 μg/mL, (*C*) 0.1 mg/mL, and (*D*) 1.0 mg/mL. The gels were run and are oriented as in Figure 1. Urocon and Urocof proteins identified by number as in Figure 1

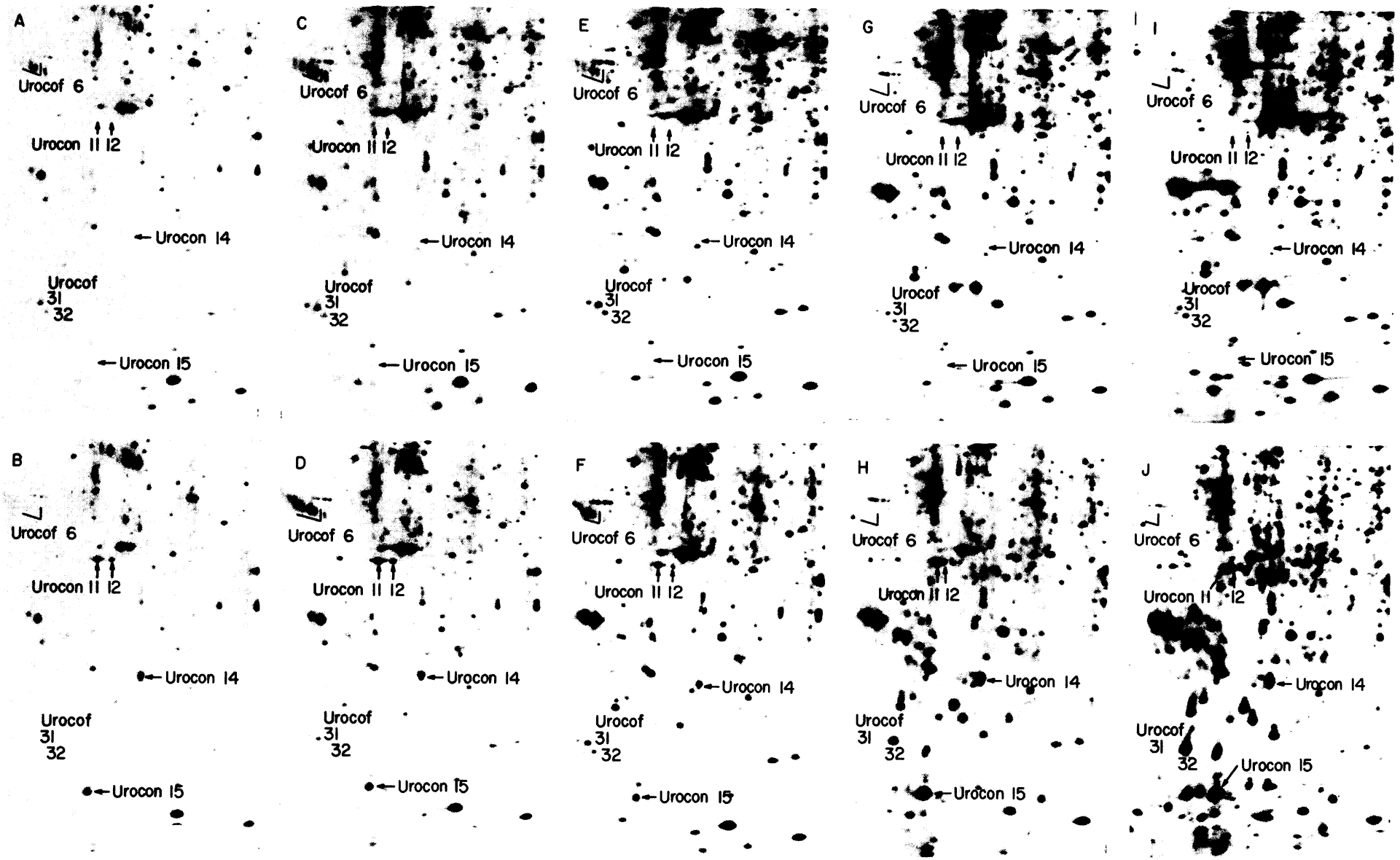

Fig. 3. Kinetic analysis of the HUP-induced changes in lymphocyte protein patterns

Sections taken from autoradiographs of human peripheral blood lymphocytes. All cells were labeled with [^{35}S]methionine in the presence or absence of 1 mg of normal HUP per milliliter for the following periods of time: (*A*) 15 min, (*B*) 15 min + HUP, (*C*) 30 min, (*D*) 30 min + HUP, (*E*) 60 min, (*F*) 60 min + HUP, (*G*) 3 h, (*H*) 3 h + HUP, (*I*) 6 h, and (*J*) 6 h + HUP. Gels run are oriented as in Figure 1. Urocon and Urocof proteins identified by number as in Figure 1

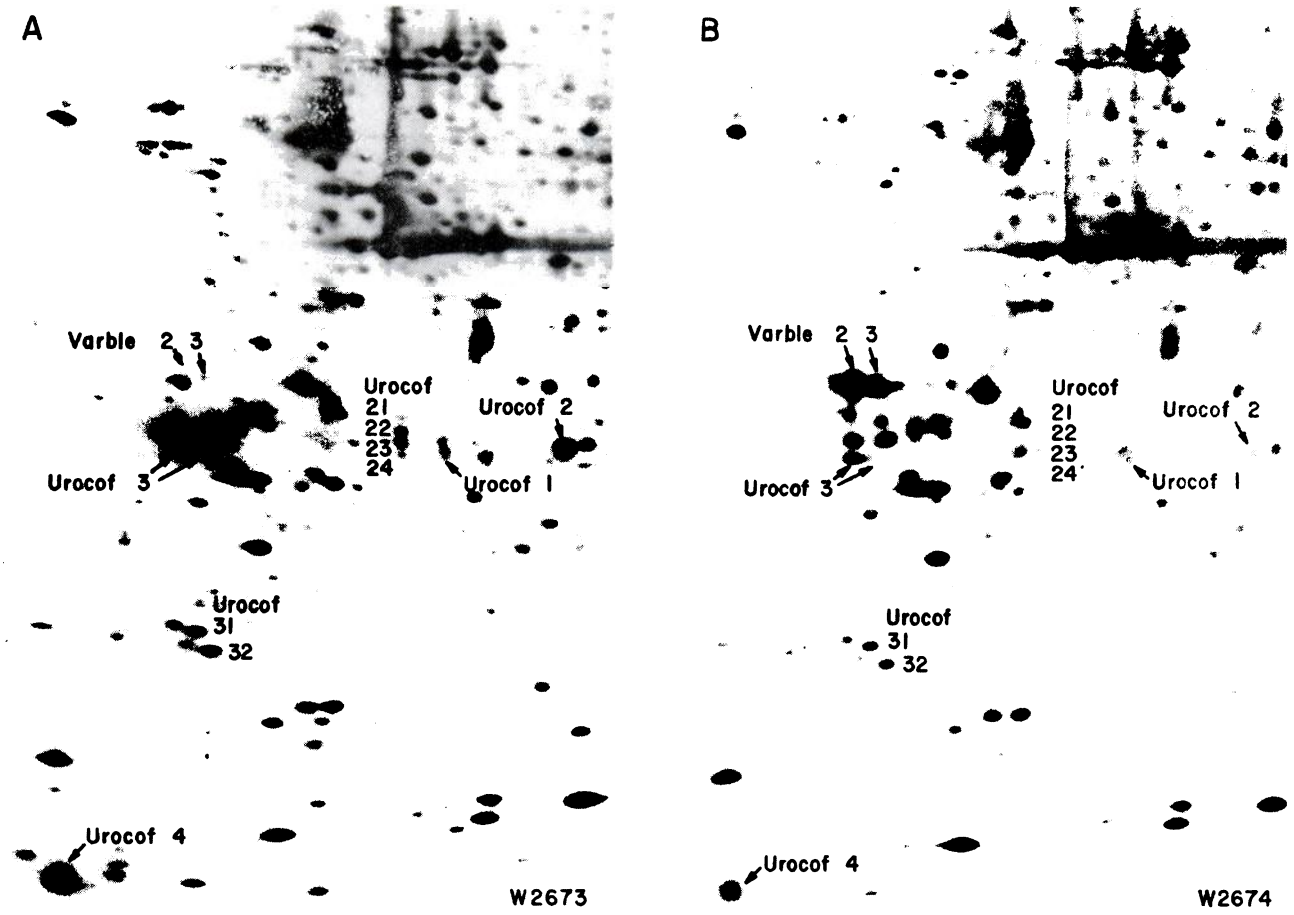

Fig. 4. Effect of human urinary histocompatibility antigens on lymphocyte protein patterns

Sections taken from autoradiographs of human peripheral blood lymphocytes from a normal 29-year-old woman. Cells were cultured (*A*) without HUP or (*B*) with 1 mg of HUP per milliliter from the same woman. Gels run and oriented as in Figure 1. Urocon and Urocof proteins identified by number as in Figure 1

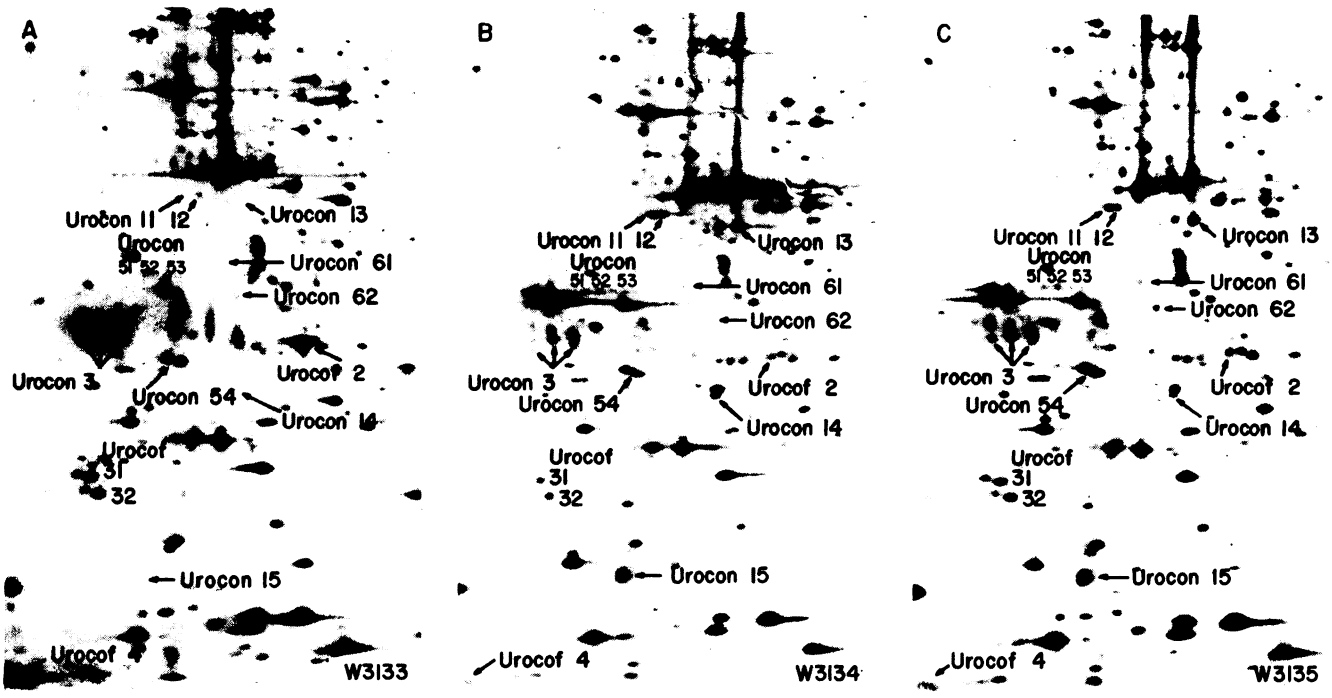

Fig. 5. Patterns of human lymphocytes treated with pathological HUP

Sections taken from autoradiographs of human peripheral blood lymphocytes. Cells were cultured (*A*) without HUP, (*B*) with 1 mg of HUP per milliliter from a man with influenza, or (*C*) with 1 mg of HUP per milliliter from a man with acute lymphocytic leukemia. Gels run and oriented as in Figure 1. Urocon and Urocof proteins identified by number as in Figure 1

spots (Figure 4) and (or) the appearance of the Urocon spots, then one would not expect to see these effects in lymphocytes treated with urinary proteins from the same individual. HUP from 12 different individuals were incubated with their own lymphocytes as well as with lymphocytes from a genetically unrelated donor; the patterns obtained were identical (Figure 4). Thus, we conclude that the alterations in lymphocyte protein patterns described here are not attributable to the specificity of the histocompatibility antigens, although this does not rule out the possibility of nonspecific effects of the HLA antigens.

Alterations in Lymphocyte Protein Synthesis by Pathological HUP

Several pathological urine samples were also analyzed for their ability to alter lymphocyte gene expression. Figure 5 shows sections of lymphocyte patterns before (*A*) and after (*B*) treatment with HUP from a patient with influenza and from a patient with acute lymphocytic leukemia (*C*). Synthesis of Urocof:1-4, 21-24, and 31-32 was diminished by treatment with either sample, but was less so in cultures treated with the leukemic-patient HUP. In addition, the presumptive cytoskeletal protein fragments (Urocon: 11-15) were present in both HUP-treated cultures (Figure 5, *B* and *C*). The run of spots labeled Urocon:51-53 (induced by urine from any patient experiencing mild to acute physical illness) and Urocon:54 were detected in both cultures; however, substantially more Urocon:54 was produced in the leukemic-HUP culture. The most interesting differences between these patterns were in the spots labeled Urocon:61-62. In the untreated lymphocyte pattern, Urocon:62 was detectable, while Urocon:61 was not. In contrast, in the patterns from lymphocytes treated with HUP from the individual with influenza, only Urocon:61 was detected; while in the cultures treated with HUP from the leukemic patient, both Urocon:61 and :62 were present.

Figure 6 shows sections taken from the high-molecular-mass acidic region of lymphocyte patterns where the cells have been treated with normal HUP (*B*) and with HUP from two renal-transplant patients (*C* and *D*). In control cultures (*A*), Urocon:71-73 were not present; however, after treatment with normal HUP (*B*), the run labeled Urocon:72 and a tiny amount of Urocon:73 were present. In Figure 6, *C* and *D*, all three Urocon proteins (:71-73) appear quite heavily labeled and show acidic charge modifications. The alterations induced by HUP from renal-transplant patients show similarities, and the quantitative differences between them are probably due to to the fact that sample C was obtained 12 days after kidney transplantation, while sample D was obtained from another patient 17 days after transplantation.

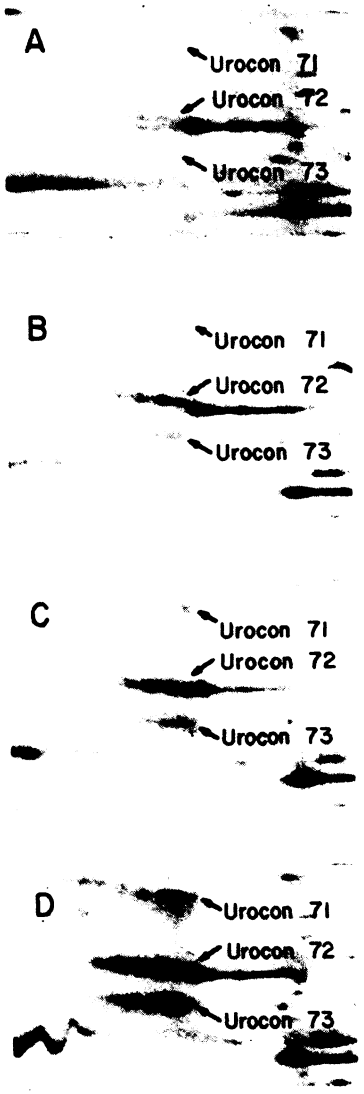

Fig. 6. Human lymphocyte protein alterations induced by HUP from renal-transplant patients

Small sections from the upper left corner of autoradiographs of human peripheral blood lymphocytes. Cells were labeled with [^{35}S]methionine and were cultured (*A*) without HUP, (*B*) with HUP from a normal man, (*C*) with HUP from a man 12 days after renal transplantation, or (*D*) with HUP from a man 17 days after renal transplantation. Gels run and oriented as in Figure 1. Urocon and Urocof proteins identified by number as in Figure 1

Discussion

The importance of understanding the functions of molecules that can regulate gene expression (including post-transcriptional control and protein processing) is becoming increasingly apparent. The biological significance of such mediators has been demonstrated by the purification and characterization of the interferons (*15*) and colony-stimulating factor (*16*). Most of the work on the interferons and colony-stimulating factor initially required large-scale purification of the molecules, and methods for obtaining large quantities of other known regulators are currently being developed in several laboratories. However, the initial detection as well as the characterization of these molecules has been limited by the biological assay systems employed. Few assays are capable of detecting activity changes at great dilutions, and even fewer are able to demonstrate a linearity of this effect (*17*). In contrast to the interferons (*18*, *19*) and colony-stimulating factor (*20*), which can be titered over a 10^6-fold range, detection of activities such as chemotactic factor (*21*), migration inhibitory factor (*22*, *23*), helper and suppressor factors (*24*, *25*), and growth factors (*26–29*) frequently becomes impossible after 10-fold dilution, and the assays fail to exhibit linear increases in activity upon concentration.

More sensitive, quantifiable, and specific assays clearly are needed for use in studying the effects of regulatory molecules. The ALE system described can establish the existence of regulators of protein gene-product expression in lymphocytes and other cells (*6*). Two-dimensional gel analysis has also been used to differentiate the in vitro effects on lymphocytes of interferon, tumor promoters, and a variety of drugs and other regulatory compounds (*30*). We demonstrate here that two-dimensional gel analysis is a reproducible, direct method for detecting changes in the cellular protein patterns produced after treatment with concentrated human urinary proteins.

The ALE system is capable of detecting biologically active molecules in human urine at low concentrations, and it has the demonstrated capability of detecting activities over a range of dilutions. Because protein detection is based on the incorporation of [^{35}S]methionine, only newly synthesized lymphocyte proteins are detected in the patterns. Experiments were performed (unpublished data) to test the effect

on human lymphocytes of various concentrations of autologous and isologous human serum as well as horse serum, bovine serum, and purified human serum components (*8*) in the culture media. None of the sera tested induced detectable alterations in the lymphocyte protein patterns. This suggests that the active factor(s) are not present in appreciable concentration in serum nor are the changes observed simply due to alterations of the protein content in the media. It is possible that lyophobic molecules such as steroids remain adsorbed to some of the urinary proteins (such as albumin) after the concentration process, and could be modulating lymphocyte protein synthesis. However, various hormones (including dexamethasone, hydrocortisone, and prednisolone) have been titrated and analyzed with this assay system, and none of the effects observed were the same as those seen with HUP-treatment of the lymphocytes (unpublished data).

Use of this technique to study HUP-regulated protein synthesis, both as a function of time and in conjunction with dose–response assays, may allow for differential analysis of heterogeneous mixtures of biologically active substances, if the effects of individual effectors are shown to be specific, and if the effectors do not produce cross inhibition. Kinetic experiments have demonstrated that the major HUP-induced effects are immediate and that they persist throughout the duration of the labeling period. Because some changes (such as in Urocof:1-4) are not readily detectable during the first few hours of incubation owing to insufficient incorporation of [^{35}S]methionine, we have chosen to use a longer labeling period to ensure efficient labeling of all the proteins. In this study, we have demonstrated that lymphocyte protein mapping can be used for the initial detection and characterization of factors that affect lymphocytes, and ultimately could be used to help determine their role in the immune response.

The central question to be examined now is whether specific sets of responses exist at the molecular level that are unique to each regulatory molecule and therefore could be used to distinguish each from all others unambiguously. To answer this question it will be necessary to map the effects of each factor individually. Thus, the mapping techniques and the effector assay (ALE) based on them make possible the discovery of effects in crude extracts or concentrates, the monitoring of isolation procedures, and ultimately the description of effects that may be unique to a specific pure substance.

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