CLIN. CHEM. 28/4, 1062–1066 (1982)

Lymphocyte, Monocyte, and Granulocyte Proteins Compared by Use of Two-Dimensional Electrophoresis

M. Anne Gemmell and N. Leigh Anderson

We compared cellular proteins from normal human blood lymphocytes, monocytes, and granulocytes, using high-resolution two-dimensional electrophoresis. The leukocytes were isolated from peripheral blood by centrifugation on density step gradients (yielding a fraction of purified granulocytes and a lymphocyte/monocyte mixture) and monocytes were subsequently separated from lymphocytes by virtue of their adherence to plastic. Wright-stained smears indicated that each of the three resulting fractions was 90 to 95% pure. The cells were labeled with [^{35}S]-methionine after various intervals in culture, then solubilized and analyzed by two-dimensional electrophoresis. Although most proteins in each cell type are common to all three, there are nevertheless several specific marker proteins that distinguish one cell type from another. We also examined the appearance of these markers in three lines of cultured cells from humans (GM607, a B-lymphoblastoid line; HL-60, a promyelocytic leukemic line; and 1494, a normal skin fibroblast).

Additional Keyphrases: *putative qualitative and quantitative marker proteins · B-lymphoblastoid cell · promyelocytic leukemia cell · fibroblast · Leukocyte Protein Index · ISO-DALT system · commonality of proteins among cell types · relative rates of protein synthesis by cell types*

The human peripheral blood leukocyte is perhaps the best place to begin the systematic mapping of human cellular proteins, because blood is the most easily and routinely available source of fresh human cells, and the leukocytes, which can easily be separated from it, can perform various interesting functions in vitro. For these reasons, we have elected to begin work towards a complete Human Protein Index (*1*, *2*) by two-dimensional electrophoretic mapping of the proteins of blood leukocytes and thereby studying their differentiational and regulatory control mechanisms (*3*, *4*). We believe that the resulting prototype data base (called the "Leukocyte Protein Index") will be useful in the study of leukemic cells, in the study of genetically determined protein variants observed in population studies, and in studies of gene regulation associated with immunological responses.

A major prerequisite for such studies is an appreciation of the major differences in gene expression among the various subtypes of leukocyte. Except for a recent study of leukocyte populations purified by cell-sorting techniques (*5*, *6*), the cells available for experimental work have generally been Ficoll–Paque purified lymphocytes (strictly speaking, "mononuclear" cells), which are usually not more than 70–90% pure. Changes in gene expression in a small proportion of these cells could therefore appear to be a general effect unless the characteristics of the subpopulations are known.

Here we have attempted to separate the three major components of the leukocyte population more cleanly, by use of their density and adherence properties, and to then identify major marker proteins of each type. The results are preliminary, in that further work, particularly of a quantitative nature [by use of the TYCHO system(*7*)], will be required to establish the relative specificities of the markers thus far identified and to identify minor ones.

Molecular Anatomy Program, Division of Biological and Medical Research, Argonne National Laboratory, Argonne, IL 60439.

Materials and Methods

Isolation and Separation of Lymphocytes, Monocytes, and Granulocytes from Peripheral Blood

The leukocytes were isolated from freshly drawn peripheral blood, collected by venipuncture into heparinized tubes (approximately 10 mL of blood per tube) from ostensibly normal persons. The whole-blood samples were centrifuged for 15 min at 500 × *g* to obtain a buffy coat, and autologous plasma was heat-inactivated at 56 °C for use in the growth medium.

Each buffy coat was diluted threefold in RPMI 1640 medium (GIBCO, Grand Island, NY 14672) and the lymphocytes, monocytes, and granulocytes were separated by gradient centrifugation in Ficoll–Paque gradients (Pharmacia Fine Chemicals, Piscataway, NJ 08854) with a cushion of 90% Percoll (Pharmacia) in phosphate-buffered saline (GIBCO). The two gradient bands (primarily lymphocytes and monocytes in the top band and granulocytes below) were washed in RPMI 1640 medium and residual erythrocytes were lysed by adding four volumes of NH_4Cl solution (8.5 g/L, pH 7.4). After erythrocyte lysis, the cells were washed in RPMI 1640 medium minus methionine (GIBCO Selectamine Kit). Cell morphology was examined under oil immersion in Wright-stained smears of the two bands, and 200 or more cells from each gradient band were counted, to estimate the purity of the cell preparations. The monocytes banded with the lymphocytes, and in five different preparations this band contained 90–95% lymphocytes plus monocytes and 5–10% granulocytes. The granulocyte band averaged 95% granulocytes and 5% lymphocytes and monocytes.

Under the culture conditions we used, the granulocytes were viable for fewer than three days (as determined by [^{35}S]methionine incorporation), whereas most of the lymphocytes were still viable at eight days (70% viable as assessed by the trypan blue exclusion technique). The leukocytes that attached to the well surface (monocyte/macrophages) did so during the 3rd to 6th day. During the first 48 h of culture these cells enlarged and formed clumps, which attached to the surface of the well. Some of these cells elongated and became fibroblastic in appearance; others took on a "poached-egg" shape. The remaining leukocytes (the lymphocytes) stayed in suspension. No attached cells were seen in the granulocyte preparations.

Culture and Labeling of Leukocytes, and Preparation of Samples

Approximately 2×10^6 cells per well were cultured in flat-bottomed multiwell plates in RPMI 1640 medium supplemented with, per liter, 200 mL of autologous human plasma, 4×10^{-5} mol of 2-mercaptoethanol, and antibiotics. They were incubated at 37 °C in a humidified atmosphere of CO_2/air (5/95 by vol). At the appropriate time, the suspended cells

were centrifuged briefly—or, if the cells were attached, the medium was aspirated, and the adherent cells were washed in RPMI-minus-methionine medium (GIBCO Selectamine Kit). Then 400 μL of labeling medium was added. The labeling medium consisted of RPMI 1640 medium minus methionine, containing, per liter, 50 mL of heat-inactivated autologous human plasma, 4×10^{-5} mol of 2-mercaptoethanol, antibiotics, and 60 mCi of [^{35}S]methionine (spec. act., 1200 kCi/mol; Amersham, Arlington Hts., IL 60005), and the cultures were incubated for 18–24 h. At the end of the labeling period, the leukocytes were harvested and solubilized in an NP-40/urea mixture as previously described (*7, 8*).

Solubilized proteins from the human lymphoblastoid B-cell line, GM607 (Human Genetic Mutant Cell Repository, Camden, NJ 08103; cultured as in ref. *9*), the human myeloid leukemic cell line HL-60 (American Type Culture Collection, Rockville, MD 20852; cultured as in ref. *9*), and the normal human skin fibroblast cell line 1494 (a gift from Meloy Laboratories, Inc., Springfield, VA 22151; NCI contract no. N01-CP91000; cultured as in ref. *10*) were analyzed in parallel with the three different leukocyte cell subpopulations.

Two-Dimensional Electrophoresis

We resolved the solubilized proteins by high-resolution two-dimensional electrophoresis, using the ISO-DALT system (*11, 12*) for the acidic and neutral proteins. The ISO gels used in the first dimension separation contained Servalyte 3.5–10 ampholytes (Serva Fine Biochemicals, Heidelberg, F.R.G.). Internal isoelectric point standards produced by carbamylation of rabbit-muscle creatine kinase (EC 2.7.3.2; Sigma Chemical Co., St. Louis, MO 63178) were used in all ISO gels (*13*). Linear gradient sodium dodecyl sulfate-slab gels (10–20% T) were used for the second-dimension separation. Gels were fixed, stained, dried, and autoradiographed or fluorographed (*14*) as described previously (*7, 8*). Three to five preparations of the three leukocyte subpopulations from different donors have been compared, with similar results in each case.

Results

The two-dimensional gels of leukocyte subpopulations shown in Figure 1 typify the significant differences we saw in gene expression among lymphocytes, monocytes, and granulocytes, even though each of the three fractions were contaminated by 5–10% with cells of other types. Granulocytes appear to differ more from lymphocytes and monocytes than these two differ from each other, as might be expected in view of the limited lifespan of the granulocyte and its consequent commitment to the production of a small number of proteins related to its specialized function. Lymphocytes and monocytes nevertheless differ in the expression of a variety of specific marker proteins.

The principal lymphocyte markers (L:1 through L:4; circles in Figure 1) are present in only trace quantities (probably the result of some contamination with lymphocytes) in monocyte and granulocyte preparations, and thus may represent true qualitative markers. Based on experiments with use of a cell sorter, it appears likely that L:1 is a T-cell marker (K. E. Willard, personal communication), rather than a general lymphocyte marker. The distribution of L:2–4 among lymphocyte subsets is currently under investigation.

The monocyte contains various specific proteins, many of which increase in relative abundance as the cells attach to the plastic of the tissue-culture vessel and become more macrophage-like. Although some of these markers (unlabeled boxes in Figure 1*C*) appear simply to be quantitatively more abundant in monocytes, some (M:1–7) appear specific enough to be considered qualitative markers. In addition, some spots identified as cell surface proteins (K. E. Willard, personal communication; S:1–4 in Figure 1*C*) are synthesized at a much higher rate in the attached monocytes than in either lymphocytes or granulocytes. A spot identified previously as a non-muscle tropomyosin (Cytosk:11 of ref. *10;* labeled *nmTm* in Figure 1*C*) is also increased in relative abundance in the attached monocytes.

The general rate of protein synthesis in granulocytes is substantially slower than that in lymphocytes or monocytes. Thus, some proteins that are produced at a high rate relative to others in the granulocyte may not in fact be produced any faster than in the monocyte, in terms of synthesis per cell. However, at least six proteins appear to be genuinely induced in the granulocyte, and to show higher absolute as well as relative rates of synthesis in these cells. They include (Figure 1*B*) the four proteins designated G:1–4 and the two proteins Calgon:1 and Calgon:2 (here labeled *C:1* and *C:2*). Calgon:1 and Calgon:2 have recently been identified as slightly different forms of the molecule described by Fagerhol as L1 (ref. *15*, and K. E. Willard, personal communication). The major mitochondrial proteins Mitcon:1–3 (ref. *9*; hexagons in Figure 1) are synthesized at substantially slower rates in the granulocyte, consistent with the limited life expectancy of this cell and the consequent lack of a need to regenerate most organelles.

Because the goal of this study is to identify candidate marker proteins for the three principal types of human blood leukocytes, it is important to investigate the appearance of these markers in the presumably more homogeneous cell populations grown as long-term-cultured cell lines. Figure 2 illustrates the results of such an investigation to survey the occurrence of the lymphocyte, monocyte, and granulocyte markers in a human B-lymphoblastoid line (GM607), a human promyelocytic leukemic line (HL-60), and a normal human fibroblast line (1494). The results, summarized in Table 1, indicate that the candidate lymphocyte markers are generally more abundant in the lymphocytic and myeloid lines. An exception is L:1, whose absence from GM607 is consistent with its identification as a T-cell marker. The monocyte, on the other hand, shares some of its markers (including an increased rate of synthesis of non-muscle tropomyosin) with the fibroblast. Some similarity between monocytes and fibroblasts might be expected because, of the cells examined here, only these two grow attached to the substrate. Nevertheless, it is interesting to note that the overproduction of surface proteins by monocytes (S:1–4) is not evident in the fibroblast; these proteins could be related to monocyte phagocytosis. The major granulocyte markers do not appear significantly in any of the cultured cell lines.

Discussion

We have identified here some protein markers characteristic of the three major types of peripheral blood leukocytes (lymphocytes, monocytes, and granulocytes) as prepared by classical physical methods. Aside from effects related to duration of culture, the results are comparable to those obtained by K. E. Willard et al. by use of cell-sorting methods (*6*; personal communication). The agreement of results by these two independent methods supports our conclusions as to the markers identified.

Information concerning cell type-specific markers is valuable in several contexts. In the first place, it allows immediate interpretation of some apparent "gene regulation" effects that in reality are the result of selective effects on subpopulations of cells. On treatment of human peripheral mononuclear cells with phorbol ester tumor promoters, for instance, several proteins are no longer seen in the two-dimensional pattern (*4*). A group of these proteins is, in fact, the set of major monocyte markers; their disappearance may thus be simply the result

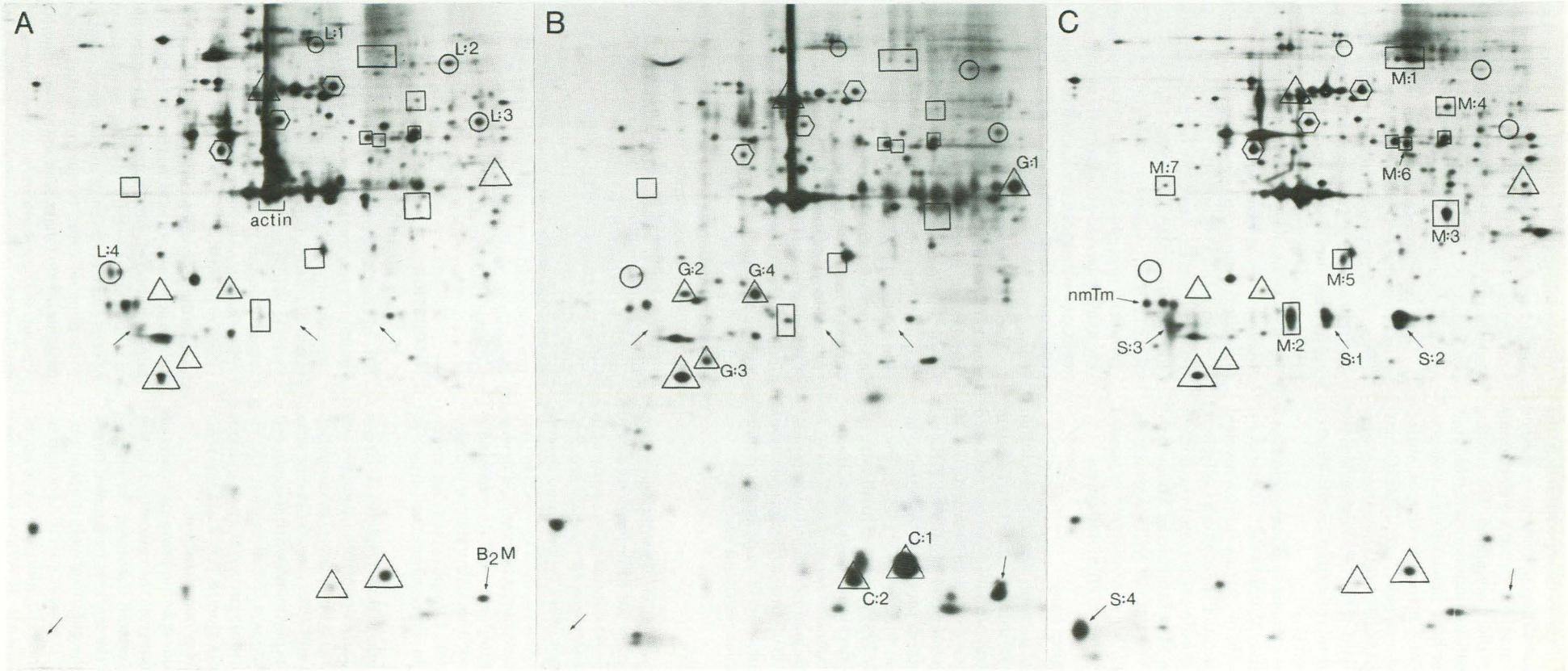

Fig. 1. Two-dimensional electrophoretic patterns of lymphocytes, granulocytes, and monocytes

(*A*) Lymphocytes (non-adherent mononuclear cells) cultured for three days, (*B*) granulocytes cultured for one day, (*C*) monocytes at day 7 of culture (having grown as attached cells for about four days). Images shown are fluorographs of gels of proteins from cells labeled with [^{35}S]methionine. The various markers include: lymphocyte-specific proteins (*L:1–4; circles*), granulocyte-specific proteins (*G:1–4, C:1–2; triangles*), monocyte-specific proteins (*M:1–7; rectangles*), monocyte surface proteins (*S:1–4*), non-muscle tropomyosin (*nmTm*), actin, and, for comparison, three major mitochondrial proteins (*hexagons*). Patterns are oriented with the acidic side to the left (basic to the right) and higher SDS-molecular masses at the top

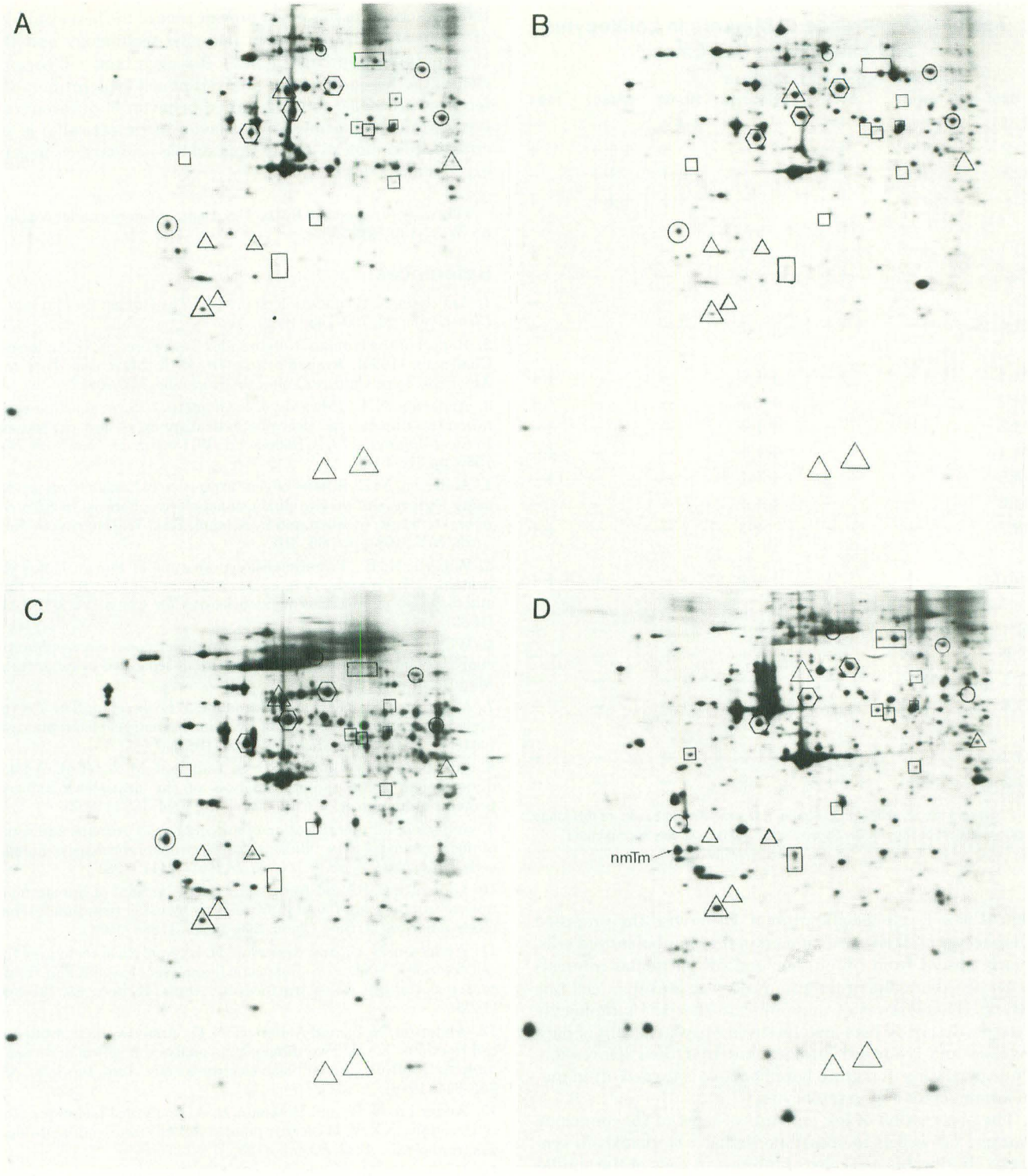

Fig. 2. Leukocyte markers in established cell lines

(*A*) Lymphocytes (as in Fig. 1*A*, but with more monocyte contamination, for comparison), (*B*) the human lymphoblastoid B-cell line GM607, (*C*) the human promyelocytic leukemic cell line HL-60, (*D*) the normal human fibroblast line 1494. Symbols indicating markers are the same as in Fig. 1

of selective toxicity of the compound in monocytes rather than a gene regulation effect in all the cells. A more important context may be the analysis of differentiation in the HL-60 cell line, where different compounds can be used to induce differentiation into either apparently mature monocytes or granulocytes (*16*). Given a spectrum of markers, it is possible to determine whether the differentiation products of this leukemic cell line are genuinely mature in all respects, and whether the markers appear with similar or different time courses during the differentiation process. Some steps have recently been taken in this direction by Liebermann et al. (*17*), though without any investigation of time course or of the properties of the cell-type-specific markers. We believe that these markers will ultimately prove useful in the analysis of human leukemic cells.

The markers are also of considerable interest in terms of their expression in the established cell lines examined here. The absence of marker L:1 from the B-lymphoblastoid cell line GM607 provides additional support for the notion (put forward by K. E. Willard, based on analogy with the mouse)

Table 1. Occurrence of Markers in Leukocytes and Established Cell Lines [a]

Spot	Lympho-cyte	Granulo-cyte	Attached monocyte	HL-60	GM607	1494
L:1	+++	+−	−	+++	−	−
L:2	+++	+−	−	+	+++	+
L:3	+++	+	−	+++	++	−
L:4	+++	+−	−	+++	+++	+
G:1	+−	+++	+	−	−	+
G:2	−	++	−	−	−	−
G:3	−	+++	−	−	−	−
G:4	+−	+++	+	+−	−	−
M:1	+−	+−	+++	−	−	++
M:2	+−	−	+++	−	−	+
M:3	+−	−	+++	−	−	+−
M:4	+	−	+++	−	−	+−
M:5	−	−	+++	−	−	+−
M:6	−	−	+++	−	−	−
M:7	−	−	++	−	−	++
nmTm	+	+	+++	+−	+	+++
S:1	+	−	+++	−	−	−
S:2	+	−	+++	−	−	−
S:3	+−	−	+++	−	−	−
S:4	+−	−	+++	+−	−	−
C:1	+	+++	++	−	−	−
C:2	+−	+++	+	−	−	−

[a] Markers are as indicated in Figure 1. Quantitation is based, at this stage, upon visual inspection of the fluorograms, and is thus only approximate.

that it is a T-cell-specific protein. Otherwise the proposed lymphocyte markers are abundant in the lymphoblastoid cells, nearly absent from fibroblasts, and (of particular interest) generally abundant in the promyelocytic leukemia cell line HL-60. This last result may indicate that the lymphocyte markers described here are present in early stem cells of both myeloid and lymphoid lineages, but that their expression, while persisting in mature lymphocytes, is turned off in mature monocytes and granulocytes.

The observation of low amounts of most of the monocyte markers (as well as increased non-muscle tropomyosin synthesis) in fibroblasts is also of interest in view of the similar attached growth habits of the two cell types. Overall, the monocyte pattern is of course much more like that of a lymphocyte than a fibroblast (in keeping with the much closer lineage relationship between them), and thus the monocyte markers (M:1–7) may be a set of proteins that is somehow involved in cell attachment. The lack of expression of the major monocyte surface proteins in fibroblasts shows, however, that not all the monocyte-specific proteins are simply related to attachment. It is interesting to speculate that these surface proteins are involved in phagocytosis, an activity at which the monocyte excels.

The identification and use of marker protein sets such as those described in this paper provides one of the best examples of the usefulness of protein mapping technology and the Protein Index approach (*1*, *3*, *4*). It is clear (and will become clearer as the quantitative analysis of cell type protein differences proceeds) that the varied behavior of different cell types (and the pathological behavior of cancer cells) is ascribable to complex—rather than simple—differences in gene expression.

This work is supported by the U.S. Dept. of Energy under contract no. W-31-109-ENG-38.

References

1. Anderson, N. G., and Anderson, N. L., The Human Protein Index. *Clin. Chem.* **28,** 739–748 (1982).

2. Report of the Human Protein Index Task Force, N. G. Anderson, Chairman, (1980). Available from Dr. R. E. Stevenson, Director, American Type Culture Collection, Rockville, MD 20852.

3. Anderson, N. L., Edwards, J. J., Giometti, C. S., et al., High-resolution two-dimensional electrophoretic mapping of human proteins. In *Electrophoresis '79*, B. Radola, Ed., W. DeGruyter, New York, NY, 1980, pp 313–318.

4. Anderson, N. L., Studies of gene expression in human lymphocytes using high-resolution two-dimensional electrophoresis. In *Electrophoresis '81*, R. C. Allen and P. Arnaud, Eds., W. DeGruyter, New York, N.Y., 1981, pp 309–316.

5. Willard, K. E., Two-dimensional analysis of human leukocyte proteins. III. Preliminary report on a marker for the early detection and diagnosis of infectious mononucleosis. *Clin. Chem.* **28,** 1031–1035 (1982).

6. Willard, K. E., and Loken, M., Two-dimensional electrophoretic analysis of human leukocyte subpopulations separated by cell sorting. Manuscript in preparation.

7. Anderson, N. L., Taylor, J., Scandora, A. E., Jr., et al., The TYCHO system for computer analysis of two-dimensional gel electrophoresis patterns. *Clin. Chem.* **27,** 1807–1820 (1981).

8. Anderson, N.L., Giometti, C. S., Gemmell, M. A., et al., A two-dimensional electrophoretic analysis of the heat-shock-induced proteins of human cells. *Clin. Chem.* **28,** 1084–1092 (1982).

9. Anderson, L., Identification of mitochondrial proteins and some of their precursors in two-dimensional electrophoretic maps of human cells. *Proc. Natl. Acad. Sci. USA* **78,** 2407–2411 (1981).

10. Giometti, C. S., and Anderson, N. L., A variant of human non-muscle tropomyosin found in fibroblasts by using two-dimensional electrophoresis. *J. Biol. Chem.* **256,** 11840–11846 (1981).

11. Anderson, N. G., and Anderson, N. L., Analytical technique for cell fractions. XXI. Two-dimensional analysis of serum and tissue proteins: Multiple isoelectric focusing. *Anal. Biochem.* **85,** 331–340 (1978).

12. Anderson, N. L., and Anderson, N. G., Analytical technique for cell fractions. XXII. Two-dimensional analysis of serum and tissue proteins: Multiple-gradient slab electrophoresis. *Anal. Biochem.* **85,** 341–354 (1978).

13. Anderson, N. L., and Hickman, B. J., Analytical techniques for cell fractions. XXIV. Isoelectric point standards for two-dimensional electrophoresis. *Anal. Biochem.* **93,** 312–320 (1979).

14. Bonner, W. M., and Laskey, R. A., A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46,** 83–88 (1974).

15. Fagerhol, M. K., Dale, I., and Andersson, T., Release and quantitation of a leucocyte derived protein (L1). *Scand J. Haematol.* **24,** 393–398 (1980).

16. Collins, S. J., Gallo, R. C., and Gallagher, R. E., Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature (London)* **270,** 347–349 (1977).

17. Liebermann, D., Hoffman-Liebermann, B., and Sachs, L., Regulation of gene expression by tumor promoters. II. Control of cell shape and developmental programs for macrophages and granulocytes in human myeloid leukemic cells. *Int. J. Cancer* **28,** 285–291 (1981).

CLIN. CHEM. 28/4, 1067–1073 (1982)

Two-Dimensional Electrophoretic Analysis of Human Leukocyte Proteins from Patients with Rheumatoid Arthritis

Karen E. Willard,[1] Anne Karine Thorsrud,[2] Eimar Munthe,[3] and Egil Jellum[2]

Human leukocyte proteins from more than 150 patients with rheumatoid arthritis, together with age- and sex-matched controls, were analyzed by use of the ISO-DALT technique of two-dimensional polyacrylamide gel electrophoresis. Patients with ankylosing spondylitis, polymyalgia rheumatica, psoriatic arthritis, calcium tendinitis, post-infectious arthritis, and asymmetrical seronegative arthritis were also included as positive controls. Synthesis of several proteins, referred to by number as members of the "Rheuma" set, is shown to increase in the leukocyte preparations from patients with classical rheumatoid arthritis. Several of these proteins are specific to monocytes or granulocytes; others are of unknown cellular origin, but appear to be unique to rheumatoid arthritis. The Rheuma proteins appear to be indicators of disease activity, because their increased synthesis can be correlated with sedimentation rate and other clinical indices of rheumatoid disease activity.

Additional Keyphrases: *the arthritides · disease of the joints · first use of ISO-DALT in a large-scale clinical study · putative "marker" proteins*

Rheumatoid arthritis is a chronic, systematic, inflammatory disease, predominantly (but not exclusively) affecting the joints. The systemic nature of the disease is characterized by an increased erythrocyte sedimentation rate, hypochromic anemia, and the appearance of characteristic anti-IgG antibodies, called "rheumatoid factors," in the serum. The sequence of immunologic events in rheumatoid arthritis seems to begin with the production of rheumatoid factors by synovial lymphocytes. These antibodies are produced during a local immune response in the joint to unknown antigens (some of which may be altered forms of IgG).

Rheumatoid factor may be found within all immunoglobulin classes, but those detected in the circulation are primarily IgM. IgG from synovial tissue and effusions of patients with rheumatoid arthritis has been shown to be present in immune complexes that initiate the local inflammatory process that causes rheumatoid disease, and to perpetuate and amplify the pathological changes that occur in the joint (*1*, *2*). Circulating rheumatoid factors are detectable in only 60–70% of patients with the disease, and a distinction is therefore made between "seropositive" and "seronegative" forms of the disease. The determination of rheumatoid factor is useful for diagnostic purposes, because there is a positive correlation between its titer in serum and disease severity. The nature of the destructive process in the joint is not entirely clear, but it is thought to be mediated by enzymes released from the lysosomes of the synovial cells, granulocytes, and macrophages in the hyperplastic synovial membrane.

Ristori et al. (*3*) analyzed membrane markers of synovial and peripheral blood lymphocytes from rheumatoid arthritis patients but were unable to detect population differences between rheumatoid arthritis patients and controls. However, using cell electrophoresis, Brown et al. (*4*) were able to determine that lymphocytes of intermediate mobility (mostly T cells) are significantly increased in the blood of patients with rheumatoid arthritis and comprise a major fraction of the lymphocyte exudate in rheumatoid synovial fluid. A peculiar but characteristic feature of rheumatoid arthritis is the increased proportion of polymorphonuclear leukocytes in the synovial fluid (*5*). The presence of immune complexes together with bound complement in the synovial membrane could provide adequate stimulus for this increase in polymorphonuclear cells, because of the chemotactic factors derived from complement activation.

The systematic analysis of approximately 1000 leukocyte proteins from patients with rheumatoid arthritis by two-dimensional polyacrylamide gel electrophoresis was undertaken to investigate the possibility of abnormal protein synthesis, post-translational modification, and (or) gene regulation in the immune cells of these individuals. Peripheral blood leukocytes were chosen for this initial study because of the routine nature of sample acquisition from both patients and controls. This analysis included blood samples from 120 volunteer patients with rheumatoid arthritis. In addition to healthy volunteer individuals, patients with ankylosing spondylitis, polymyalgia rheumatica, psoriatic arthritis, tendinitis, post-infectious arthritis, and asymmetrical seronegative arthritis were included as positive controls. The two-dimensional gel analysis was performed without knowledge of the clinical diagnosis or review of patients' test results; however, after the initial grouping the interpretation of the data was correlated with clinical findings. This is the first large-scale clinical study performed with use of the ISO-DALT technique (*6*, *7*) for two-dimensional gel analysis. A group of proteins, referred to as the "Rheuma set," appear to be indicators of rheumatoid arthritis disease activity.

Materials and Methods

Isolation of Leukocytes

All experiments were performed on human peripheral blood leukocytes from patients with classical rheumatoid arthritis (Am. Rheumatism Assoc. criteria), ankylosing spondylitis, polymyalgia rheumatica, psoriatic arthritis, tendinitis, post-infectious arthritis, or asymmetrical seronegative arthritis, and from normal donors. The patients were all under the care of a rheumatologist in Oslo, Norway.

Peripheral blood (10 mL) was collected by venipuncture into heparinized tubes, equilibrated on a rocker panel until all samples were collected, and then centrifuged for 15 min at $500 \times g$ to obtain buffy coats. Buffy coat leukocytes were diluted threefold in RPMI 1640 medium (Flow Laboratories, Irvine KA12 8NB, Scotland), and the lymphocyte fraction (also containing monocytes and a small percentage of contaminating granulocytes) was isolated by Ficoll-Paque gradient centrifugation (Pharmacia Fine Chemicals AB, Uppsala,

[1] Molecular Anatomy Program, Division of Biological and Medical Research, Argonne National Laboratory, Argonne, IL 60439.
[2] Institute of Clinical Biochemistry, Rikshospitalet, University of Oslo, Oslo 1, Norway.
[3] Oslo City Department of Rheumatology, Diakonhjemmets Hospital, Oslo 3, Norway.

Sweden). Enriched lymphocyte bands (referred to here as leukocytes) were washed twice in RPMI 1640 medium and then finally washed in RPMI 1640 containing no methionine (Select-amine kit; GIBCO, Grand Island, NY 14672).

Labeling of Leukocyte Proteins

Leukocytes were cultured in flat-bottomed, multi-well plates at concentrations of up to 4×10^6 cells per well in a total volume of 400 μL. Cells were cultured in RPMI 1640 labeling medium (which contains no methionine) supplemented with fetal bovine serum (50 mL/L), 2-mercaptoethanol (4×10^{-5} mol/L), and 25–50 μCi of [^{35}S]methionine (spec. acty. 1420 kCi/mol; Amersham International Ltd., Amersham, Buckinghamshire HP7 9LL, England). Cultures were incubated at 37 °C in a humidified atmosphere containing five volumes of CO_2 per 100 volumes, for 18 h unless otherwise indicated.

Two-Dimensional Polyacrylamide Gel Electrophoresis

At the end of the labeling period, leukocytes were harvested by brief centrifugation in a microcentrifuge (Beckman Microfuge B; Beckman Instruments, Palo Alto, CA 94304), and the cell pellets were lysed in a buffer containing, per liter, 40 mL of Nonidet P40 detergent, 9 mol of urea, 20 mL of ampholytes (Ampholines; LKB Instruments, Bromma, Sweden), and 50 mL of 2-mercaptoethanol. Solubilized samples were centrifuged in the microcentrifuge for 1 min to sediment the insoluble material. The soluble proteins were analyzed by high-resolution two-dimensional electrophoresis (ISO-DALT system, *6*, *7*) to resolve the acidic and neutral proteins. The ISO gels used in the first-dimension separation contained pH 3.5–10 Ampholines (LKB Instruments). Internal isoelectric-point standards, produced by carbamylation of rabbit muscle creatine kinase (EC 2.7.3.2; Sigma Chemical Co., St. Louis, MO 63178), were used in all ISO gels (*8*). Second-dimension separations were perfomed on a linear-gradient 100–200 g/L polyacrylamide gel. Gels were fixed, dried, and autoradiographed for one to two weeks as described previously (*9*).

At least two normal controls were used every time a patient's blood samples were examined.

Results

Alterations in Leukocyte Protein Synthesis in Rheumatoid Arthritis

Figure 1 illustrates the alterations detected in two-dimensional gel autoradiographs of human leukocyte proteins from patients with rheumatoid arthritis. Figure 1*A* shows an autoradiograph of leukocyte proteins from a normal individual, and Figures 1*B* and 1*C* represent typical rheumatoid patterns. The synthesis of several proteins (determined by the incorporation of [^{35}S]methionine) increases, and some proteins not seen before appear in the leukocyte preparations from patients with rheumatoid arthritis. These proteins (indicated in Figure 1 by the letter R) are referred to by number as members of the "Rheuma" set under the set-naming conventions discussed previously (*10*).

Rheuma:1 has been shown to be a monocyte marker protein by fluorescence-activated cell sorting with use of the monoclonal antibody OKM.1 and subsequent two-dimensional gel analysis of the purified monocyte proteins (K. E. Willard et al., manuscript in preparation).

Gel analysis of granulocytes purified on the basis of right-angle light scattering by use of the cell sorter indicates that Rheuma:3 is a major granulocyte marker protein, although small amounts can be detected in monocytes as well.

Rheuma:4 and Rheuma:5 have not been detected before this analysis of rheumatoid arthritis leukocyte proteins, and therefore may be unique markers of the disease. It is not possible to assign a cell lineage to the origin of Rheuma:4–5 until leukocytes from patients with active rheumatoid arthritis can be purified by using fluorescence-activated cell sorting analysis.

Rheuma:6–7 are present in granulocytes, monocytes, and lymphocytes; however, their relative abundances vary: granulocytes contain more than ten times the quantity in monocytes, and monocytes more than twice the amount present in lymphocytes. Rheuma:6–7 are phosphorylated, as demonstrated by ^{32}P-labeling studies (N. Leigh Anderson, personal communication). A collaborative analysis has also revealed that Rheuma:6–7 are the same as the L_1 protein described by Fagherol et al. (*11*, *12*).

The leukocyte proteins from the two patients with active rheumatoid arthritis (Figures 1*B* and 1*C*) are representative of the pattern seen for this disease as compared with an age-matched control (Figure 1*A*). Patient OS-2 (Figure 1*B*) is a 56-year-old woman who had not responded to treatment. At the time the blood sample was taken the sedimentation rate was 110 mm/h and the leukocyte count was 14 500/mm^3. She had strong seropositive rheumatoid arthritis with a Waaler–Rose titer of 1000. At the time of our analysis her condition was deteriorating, and it did not improve when she was treated with penicillamine.

Patient OS-5 (Figure 1*C*), a 69-year-old man, was first diagnosed with rheumatoid arthritis one year before the blood sampling for two-dimensional gel analysis. At the time of sample acquisition the sedimentation rate was 82 mm/h and the leukocyte count was 7900/mm^3. He also has strong seropositive rheumatoid arthritis with a Waaler–Rose titer of 2000. Therapy with prednisolone (30 mg/day) and D-penicillamine (250 mg/day) was begun one month before our sampling, but the patient was only beginning to respond to these drugs at the time of our sampling.

Comparison of Leukocyte Proteins from a Variety of Arthritis Patients

Figure 2 shows sections of two-dimensional gel autoradiographs of leukocytes from a normal donor (*A*) and patients with classical rheumatoid arthritis (*B*), post-infectious arthritis (*C*), ankylosing spondylitis (*D*), polymyalgia rheumatica (*E*), and psoriatic arthritis (*F*).

Patient OS-40 (Figure 2*B*), a 60-year-old man, had just started to respond to treatment with gold thiomalate (Myocrisin R; May and Baker, Inc., England) at the time our sample was taken for analysis. The sedimentation rate was 57 mm/h and the leukocyte count was 7000/mm^3. The patient had seropositive rheumatoid arthritis, with a Waaler–Rose titer of 250 and he demonstrated the increased amounts of Rheuma:1, :3, and :6–7 that seem to be characteristic of classical rheumatoid arthritis. In addition, the markers Rheuma:4–5 are also present in this leukocyte pattern, as they were in all samples from patients with active rheumatoid arthritis.

Although a definitive diagnosis was not possible on patient OS-38 (Figure 2*C*), a 24-year-old woman, the tentative diagnosis is post-infectious arthritis. This patient demonstrated arthritis in one knee and had cystitis in the initial stages of the disease, which began with general anorexia and joint pain. The sample taken for two-dimensional gel analysis was acquired early in the disease when the sedimentation rate was 33 mm/h. She was seronegative to the Waaler–Rose test. The protein pattern shows a slight increase in Rheuma:1, :3, and :6–7 over the control sample (but less than half the increase seen in OS-40). In addition, a small amount of Rheuma:4 was detected. Rheuma:5 is diminished in this protein pattern.

Patient OS-41 (Figure 2*D*), a 32-year-old man, has ankylosing spondylitis, a chronic inflammatory disease affecting

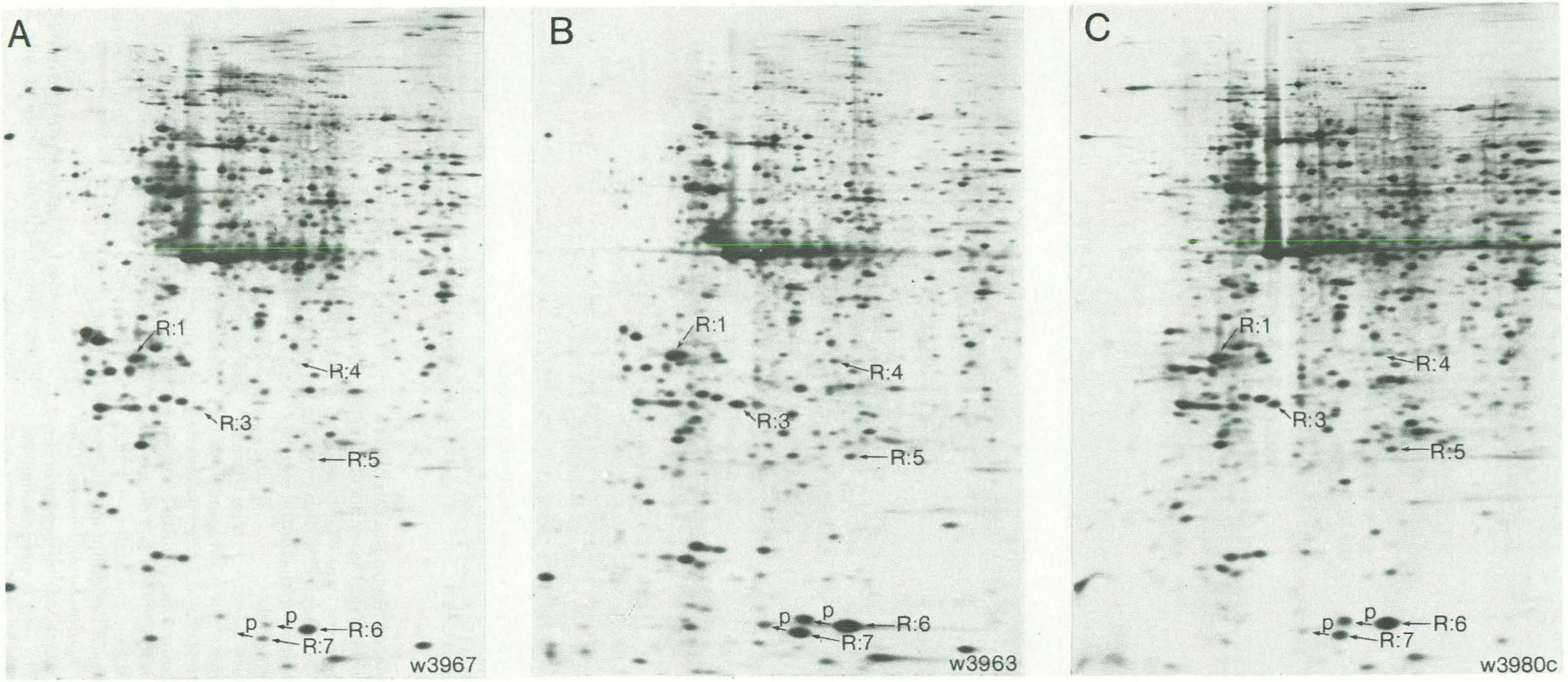

Fig. 1. Leukocyte protein patterns from patients with rheumatoid arthritis

Peripheral blood leukocytes were radiolabeled with [^{35}S]methionine for 18 h. In all these figures, the gels are oriented with the basic end of the ISO gel to the right and the 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. Sections from autoradiographs shown here: (*A*) normal individual without arthritis, (*B*) rheumatoid arthritis Patient OS-2, and (*C*) rheumatoid arthritis Patient OS-5. The Rheuma (*R*) proteins are identified by number

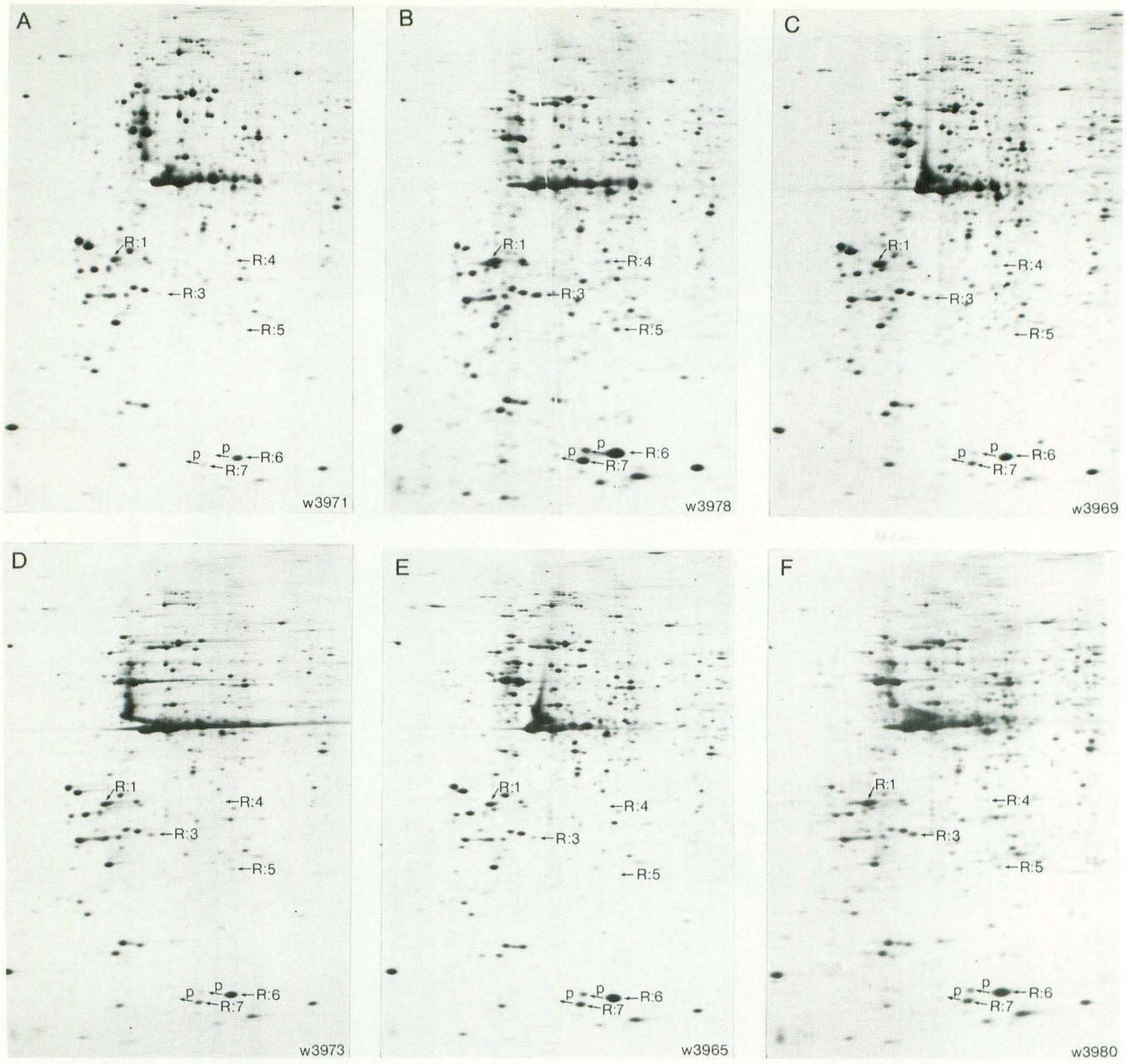

Fig. 2. Leukocyte protein patterns from a variety of arthritis patients

Sections taken from autoradiographs of human peripheral blood leukocytes from: (*A*) normal individual without arthritis, (*B*) rheumatoid arthritis Patient OS-40, (*C*) post-infectious arthritis Patient OS-38, (*D*) ankylosing spondylitis Patient OS-41, (*E*) polymyalgia rheumatica Patient OS-30, and (*F*) psoriatic arthritis Patient OS-37. Rheuma proteins are identified as in Figure 1

the sacroiliac joints and the spine. At the time of sample acquisition for two-dimensional gel analysis, the sedimentation rate was 10 mm/h and the leukocyte count was 4000/mm^3. The relative amounts of Rheuma:1, :3, and :6–7 in this protein pattern are essentially identical to those in the control pattern, and Rheuma:4–5 were barely detectable.

Figure 2*E* shows the pattern for a 71-year-old man (Patient OS-30) with polymyalgia rheumatica, a poorly understood muscular disease. This condition is characterized by muscle pain with the absence of inflammatory arthritis, but the erythrocyte sedimentation rate is much increased during the active phase. Patient OS-30 had been responding to a high dose of prednisolone, which relieved the symptoms and decreased the sedimentation rate to near-normal values. The sample taken for analysis was obtained after the dose of corticosteroids had been decreased to 5 mg of prednisolone per day and the sedimentation rate had declined to 12 mm/h. There was no apparent difference between the leukocyte protein pattern obtained for Patient OS-30 and that for the normal control sample. Rheuma:4–5 were not present in his pattern, and the relative amounts of Rheuma:1, :3, and :6–7 were approximately the same as in the normal donor (Figure 2*A*).

Patient OS-37 (Figure 2*F*) was a 22-year-old man with seronegative psoriatic arthritis. At the time the blood sample was taken, the sedimentation rate was 36 mm/h and the leukocyte count was 8000/mm^3. Comparison of his two-dimensional leukocyte pattern with an age-matched control demonstrated that the amounts of Rheuma:1, :3, and :6–7 were not significantly increased and that Rheuma:4–5 were not detectable.

Comparison of Leukocyte Protein Patterns from Rheumatoid Arthritis Patients with Active and Inactive Disease

The Rheuma set of leukocyte proteins thus appear to be

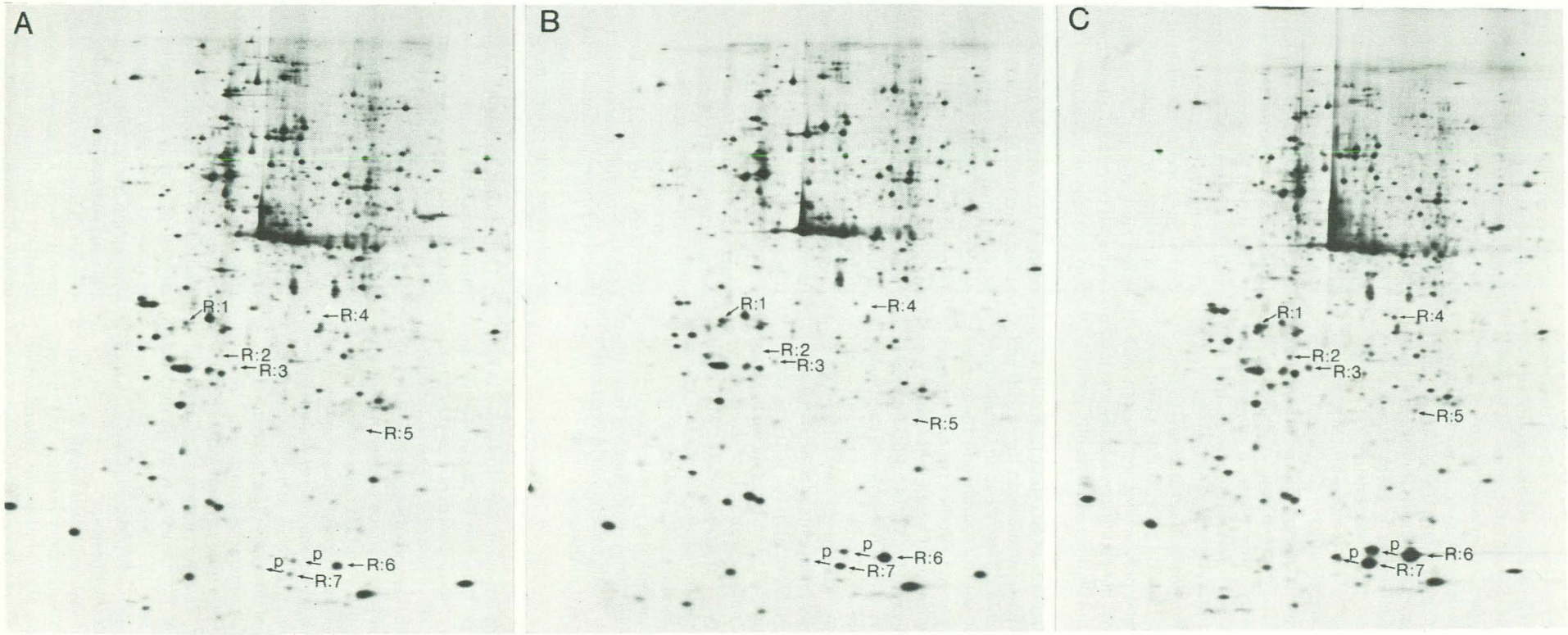

Fig. 3. Leukocyte protein patterns from rheumatoid arthritis patients with active and inactive disease

Relevant portions of autoradiographs of human peripheral blood lymphocytes from (*A*) normal individual without arthritis, (*B*) inactive rheumatoid arthritis Patient OS-24, and (*C*) classical rheumatoid arthritis Patient OS-23. Rheuma proteins are identified as in Figure 1

indicators of rheumatoid arthritis disease activity, and therefore we compared two patients, one with active rheumatoid arthritis and one whose disease had improved and stabilized on penicillamine (Figure 3). Figure 3*A* shows a section from a two-dimensional gel autoradiograph of leukocyte proteins from a normal donor. Figure 3*B* shows the pattern for an 83-year-old woman (Patient OS-24) who has had classical symptoms of rheumatoid arthritis for longer than 30 years. She improved on penicillamine in recent years and was in a stable condition with very little disease activity at the time the sample illustrated was taken for two-dimensional gel analysis. At this time, the sedimentation rate was 37 mm/h and the leukocyte count was 7400/mm^3. On the other hand, Patient OS-23 (Figure 3*C*), a 57-year-old woman, had classical rheumatoid arthritis in a very active phase at the time the sample was collected. She has seropositive rheumatoid arthritis, with a Waaler–Rose titer of 300. At the time the blood sample was taken for two-dimensional gel analysis, the sedimentation rate was 80 mm/h and the leukocyte count was 15 400/mm^3. The patient had previously been treated with gold, but it had not improved her condition, and she was just beginning a course on D-penicillamine when we obtained the sample.

The differences in the amounts of Rheuma:1, :3, and :6–7 are readily apparent in the patterns shown in Figures 3*B* and 3*C*. There is at least a twofold difference in the quantity of these proteins between the control sample and the patient with inactive disease (OS-24) and another twofold increase in the amount of protein synthesized by the leukocytes from the patient with active disease (OS-23). In addition, only in Patient OS-23 were Rheuma:4–5 detectable, strengthening the argument that these are novel proteins in patients with active rheumatoid arthritis. Rheuma:2, like Rheuma:4–5, appears to be detectable only in patients with active disease. Although Rheuma:2 was not detected in the patterns shown in Figures 1 and 2, it was found in the patterns shown in Figure 3. The patterns in Figures 1 and 2 were obtained (by the same individuals) with the ISO-DALT apparatus in the Molecular Anatomy Program at Argonne; the patterns in the third figure were obtained with the commercial version of the ISO-DALT apparatus, at the Rikshospitalet in Oslo. We attribute the apparent shift in the molecular mass of Rheuma:3 and the appearance of Rheuma:2 to subtle differences in the brands of sodium dodecyl sulfate used in the gels.

Discussion

It has become increasingly apparent that alterations in the regulation or synthesis of proteins can be implicated in the pathogenesis of a variety of clinical disorders. It is possible to study the synthesis of hundreds of proteins or protein subunits by radiolabeling cellular proteins and subsequently analyzing them by two-dimensional polyacrylamide gel electrophoresis (*13*). It has also been demonstrated that such a two-dimensional analysis can be used to study regulation events within the cell (*10*, *14*). Recent investigations have revealed differences in the two-dimensional patterns of leukocytes from patients with infectious mononucleosis (*15*), leukemia (K. E. Willard et al., manuscript in preparation), intestinal biopsies (*16*), and muscle biopsy samples from patients with a number of muscular diseases (*17*). Thus we undertook systematic analysis of leukocytes from 120 patients with rheumatoid arthritis and 30 controls, with the idea of surveying their protein patterns for abnormally synthesized proteins, which might then be implicated in the disease.

The purpose of this large-scale protein analysis of cells involved in the rheumatoid inflammatory reaction was to find indicator proteins that are either abnormally abundant or diminished in leukocyte patterns from patients with active disease. Ultimately, these target proteins might be used as markers in the diagnosis of rheumatoid arthritis by two-dimensional gel analysis or other techniques such as radioimmunoassay. However, the technique has not yet been automated or mechanized to the point where it can be used as a routine clinical tool, nor has a sufficiently large data base been built to support the diagnosis of human disease by two-dimensional gels. In addition, alterations in the relative numbers of granulocytes that sediment with the lymphocytes and monocytes in the top layer of a Ficoll-Paque gradient can affect results of this type of analysis. However, at the present time, two-dimensional gels are an excellent research tool and as such provide a means for targeting proteins that might be implicated in a particular disease.

The initial gel screen was undertaken as a blind study without the analysts' knowledge of the clinical diagnosis or a review of the patients' test results. This allowed us, first to derive our conclusions without prejudice and subsequently to correlate our findings with conclusions drawn from established clinical criteria. The results described here have demonstrated consistent alterations in the two-dimensional protein patterns obtained from Ficoll-Paque-isolated leukocyte populations of rheumatoid arthritis patients. These proteins, referred to by number as members of the Rheuma set, increase at least fivefold in patients with active rheumatoid arthritis as compared with patients with ankylosing spondylitis, polymyalgia rheumatica, psoriatic arthritis, tendinitis, or asymmetrical seronegative arthritis. Patterns from patients with post-infectious arthritis often show slightly increased amounts of the Rheuma proteins, implying that an increase in these proteins may be characteristic of inflammatory arthritis. In accordance with this, patients with polymyalgia rheumatica who have a high sedimentation rate but no inflammatory arthritis do not have increased amounts of the Rheuma proteins.

The analysis of leukocyte subpopulations by fluorescence-activated cell sorting coupled with two-dimensional polyacrylamide gel electrophoresis (K. E. Willard et al., manuscript in preparation) has demonstrated that Rheuma:1 is a monocyte protein, while Rheuma:3 and :6–7 are found in both monocytes and granulocytes, with a greater abundance of these proteins characteristic of granulocytes. The cellular origin of Rheuma:2 and :4–5 has not been definitively determined by our initial analysis of the human leukocyte subpopulations, but preliminary data suggest that they are of monocytic origin.

Differential leukocyte counts on peripheral blood from rheumatoid arthritis patients did not differ significantly from the controls (averaging about 6% monocytes, 50% granulocytes, and 30% lymphocytes in both). Therefore, it is unlikely that the increase in the Rheuma proteins is simply due to a general increase in monocytes or granulocytes in the peripheral blood. Future analyses will include differential counts on the harvested cell populations to determine the exact ratio of monocytes, granulocytes, and lymphocytes in the cell populations being labeled. The increase in granulocyte proteins could be caused by alterations in the cells themselves that affect their behavior on a Ficoll-Paque gradient and increase the number of granulocytes contaminating the top layer of the gradient. Such contamination would not account for the increased synthesis of monocyte-specific proteins, because monocytes normally sediment with lymphocytes in our gradients, and the number of monocytes in the blood of arthritis patients was not seen to differ significantly from control values. An alternative explanation lies in the nature of the disease process itself. Although rheumatoid arthritis is not well understood, it is thought that monocyte, granulocyte, and synovial cell enzymes are released in the joint and mediate its

destruction. An increase in the synthesis of specific proteins from these cell types could be a reflection of this phenomenon.

The work described here is presented as a preliminary analysis of rheumatoid arthritis with two-dimensional gels, to determine whether altered protein synthesis in patients' leukocytes could be implicated in the disease. Future work must include an analysis of purified leukocyte subpopulations from patients and controls with use either of cell sorting or in vitro culturing techniques, to examine directly the specificity of the Rheuma proteins as well as to determine whether their synthesis is being selectively regulated as a manifestation of the disease. In addition, this type of analysis would provide information about the cellular origin of Rheuma:2 and :4–5 and further evidence to determine whether they are in fact characteristic of leukocytes from patients with rheumatoid arthritis or perhaps represent more general inflammatory arthritis.

We thank Dr. Norman G. Anderson for his cooperation in this collaborative project. In addition, we thank all the members of the Molecular Anatomy Program at Argonne and the Institute for Clinical Biochemistry at the Rikshospitalet for their helpful discussions. The cooperation of the patients is also greatly appreciated. This work was supported by grants from Norsk Hydro Norske Kvinners Sanitetsforening, Trondheim; Det Norske Reumatismeråd, NATO (R.G.266.80); and the U.S. Dept. of Energy under contract no. W-31-109-ENG-38.

References

1. Munthe, E., and Natvig, J. B., Immunoglobulin classes, subclasses, and complexes of IgG rheumatoid factor in rheumatoid plasma cells. *Clin. Exp. Immunol.* **12,** 55–70 (1972).

2. Male, D., Rott, J. M., and Hay, F. C., Analysis of immune complexes in synovial effusions of patients with rheumatoid arthritis. *Clin. Exp. Immunol.* **39,** 287–306 (1980).

3. Ristori, J. M., Sauvezie, B., Chollet, P., et al., Lymphocyte populations in the blood and synoviàl fluid in rheumatoid polyarthritis. Importance of cell electrophoresis. *Rev. Rhum. Mal. Osteo-Articulaires* **46,** 475–481 (1979).

4. Brown, K. A., Embling, P. H., Perry, J. D., and Holbrow, E. J., Electrophoretic behavior of blood and synovial fluid lymphocytes in rheumatoid arthritis. *Clin. Exp. Immunol.* **36,** 272–278 (1979).

5. Cochrane, S., The role of immune complexes and complement in tissue injury. *J. Allergy* **42,** 113 (1968).

6. Anderson, N. G., and Anderson, N. L., Analytical technique for cell fractions. XXI. Two-dimensional analysis of serum and tissue proteins: Multiple isoelectric focusing. *Anal. Biochem.* **85,** 331–340 (1978).

7. Anderson, N. L., and Anderson, N. G., Analytical technique for cell fractions. XXII. Two-dimensional analysis of serum and tissue proteins: Multiple gradient-slab electrophoresis. *Anal. Biochem.* **85,** 341–354 (1978).

8. Anderson, N. L., and Hickman, B. J., Analytical techniques for cell fractions. XXIV. Isoelectric point standards for two-dimensional electrophoresis. *Anal. Biochem.* **93,** 312–320 (1979).

9. Willard, K. E., and Anderson, N. G., Alterations in gene expression in Novikoff hepatoma cells induced by a factor in human urine. *Biochem. Biophys. Res. Commun.* **91,** 1089–1094 (1979).

10. Willard, K. E., and Anderson, N. G., Two-dimensional analysis of human lymphocyte proteins: I. An assay for lymphocyte effectors. *Clin. Chem.* **27,** 1327–1334 (1981).

11. Fagerhol, M., Dale, I., and Andersson, T., Release and quantitation of a leucocyte derived protein (L1). *Scand. J. Haematol.* **24,** 393–398 (1980).

12. Fagerhol, M. K., Dale, I., and Andersson, T., A radioimmunoassay for a granulocyte protein as a marker in studies on the turnover of such cells. *Bull. Eur. Physiopathol. Resp.* **16,** 273–281 (1980).

13. Anderson, N. G., and Anderson, N. L., Molecular anatomy. *Behring Inst. Mitt.* **63,** 169–210 (1979).

14. Anderson, L., Identification of mitochondrial proteins and some of their precursors in two-dimensional electrophoretic maps of human cells. *Proc. Natl. Acad. Sci. USA* **78,** 2407–2411 (1981).

15. Willard, K. E., Two-dimensional analysis of human leukocyte proteins. III. Preliminary report on a marker for the early detection and diagnosis of infectious mononucleosis. *Clin. Chem.* **28,** 1031–1035 (1982).

16. Thorsrud, A. K., Vatn, M. H., and Jellum, E., Two-dimensional electrophoretic patterns of normal mucosa, polyps, and carcinomas of the large intestine. *Clin. Chem.* **28,** 884–889 (1982).

17. Giometti, C. S., Barany, M., Danon, M. J., and Anderson, N. G., Muscle protein analysis. II. Two-dimensional electrophoresis of normal and diseased human skeletal muscle. *Clin. Chem.* **26,** 1152–1155 (1980).