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Human leukemias: A preliminary 2-D electrophoretic analysis*

We have investigated the qualitative and quantitative differences in the two-dimensional (2-D) gel fluorographic patterns of biosynthetically pulse-labeled cellular proteins from the malignant cells of patients with various leukemias (acute myelogenous, acute lymphoblastic, chronic lymphocytic, and hairy cell). Significant similarities and differences between the major types of leukemias were found. An initial small data base was constructed using our GELLAB computer analysis system for 2-D gels in order to define methods for data base definition, normalization, and visual verification of results. Search strategies to find significant spot differences, both qualitative and quantitative, between currently accepted classes of leukemias were explored. Potential methods of expressing in a summary statistic the overall similarities and differences between gels were also investigated. A series of proteins whose relative synthetic rates may serve as potential markers of states of differentiation in human leukemias were identified in this initial data base and are reported here.

1 Introduction

It is axiomatic to the practice of medicine that the appropriate choice of therapy requires precision in diagnosis and prognosis. This is particularly true in the care of patients with leukemia. Much current research in hematology has centered on the development of diagnostic methods capable of characterizing the phenotypic differentiation of the malignant cell populations comprising the various types of leukemias. Even within a relatively narrow diagnostic subclass (*e. g.* acute myelomonocytic leukemia) the wide heterogeneity of clinical and biological behavior and prognosis demands a more meaningful scheme than the current French-American-British (FAB) classification [1]. The protein composition of the malignant cells in both its qualitative and quantitative aspects reflects the genetic expression, or differentiation, occurring in that population. Thus, we have been interested in utilizing a precise analysis of those proteins displayed with 2-D gel electrophoresis to distinguish the state of differentiation of that population. Information derived from other current diagnostic methods such as enzyme histochemistry or cell surface markers, including reactivity with monoclonal antibodies, should largely be implicit in the 2-D gel patterns of leukemic cell proteins. Likewise, additional information of potential prognostic significance, such as cell cycle kinetic data, may also be determined by analysis of specific sets of proteins.

We have previously shown that 2-D gels of whole cell lysates of biosynthetically radiolabeled proteins of normal human peripheral blood lymphocytes may be used to identify proteins whose relative synthetic rates serve as markers of growth activation [2]. As a preliminary experiment to evaluate the diagnostic potential of 2-D gels for distin-

guishing differing states of differentiation, we have compared the 2-D gel patterns of proteins from a pair of autologous human lymphoblastoid cell lines, one of T and the other of B cell origin, and found significant differences [3]. We, and others, have also begun to examine the 2-D patterns of freshly isolated leukemic cells [3, 4]. In the course of these investigations, it became apparent that the massive amounts of data contained in 2-D gel images would require computer-assisted techniques for optimal analysis. For this purpose, we have developed the GELLAB system which is capable of accurately locating and quantifying the majority of spots in a 2-D gel image, comparing similar gel images, and constructing a unified data base consisting of many images suitable for statistical analysis [5-8]. We report here the next step in the application of these principles to the problem of clinically relevant distinctions between the various forms of human leukemia. We have constructed an initial data base consisting of 2-D gel fluorographs of a small number of samples of leukemic cells representative of common forms of leukemia. We have sought and found features in the gel patterns which allow the distinction of the acute myeloid leukemia (AML) samples from those from the lymphoid leukemias - chronic lymphocytic leukemia (CLL), hairy cell leukemia (HCL), or leukemic reticuloendotheliosis, and acute lymphoblastic leukemia (ALL). Furthermore, within each of these standard diagnostic categories, considerable heterogeneity of the 2-D gel patterns has been demonstrated. Such heterogeneity promises even greater diagnostic and prognostic potential.

2 Methods

2.1 Leukemic cell isolation and biosynthetic pulse-labeling

Leukemic cells were isolated by Ficol-Hypaque density gradient centrifugation, from 50 ml samples of peripheral blood

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Abbreviations: 2-D: Two-dimensional; FAB: French-American-British; AML: Acute myelogenous leukemia; ALL: Acute lymphoblastic leukemia; CLL: Chronic lymphocytic leukemia; HCL: Hairy cell leukemia; IPS: Image processing section; WRS: Wilcoxon rank sum

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from previously untreated patients with AML, ALL, CLL or HCL, as previously described [3]. Wright-stained cytocentrifuge preparations of the isolated cells were examined by light microscopy to ensure that > 95 % of the cells were members of the malignant (leukemic) cell population. Freshly isolated cells in aliquots of 20×10^6 cells were washed once with Hank's balanced salt solution and immediately biosynthetically radiolabeled with [^3H]-leucine in leucine-deficient RPMI-1640 for 2 h, as previously described [3]. After labeling, the cells were washed twice in cold phosphate-buffered saline and suspended in 200 μl of lysis buffer (9 M urea, 2 % NP-40, 5 % mercaptoethanol, 2 % LKB carrier ampholytes, pH 3.5-10, 1 mM phenylmethylsulfonylfluoride). Lysis buffer was titrated to a pH > 9.0 before use.

2.2 Electrophoresis and fluorography

Forty μl aliquots of whole-cell extracts in lysis buffer were subjected to 2-D gel electrophoresis using the Iso-Dalt apparatus, as previously described [3]. Gels were stained with Coomassie Blue, subjected to fluorography using 4 % 2,5-diphenyloxazole in glacial acetic acid [3], and exposed to Kodak XAR-5 film for varying periods of time (96-720 h, a function of markedly varying total protein synthetic rates in different patient samples) to obtain exposures of approximately equal total density.

2.3 Computer analysis with GELLAB

Fluorographic images on film were digitized into 512×512 arrays of gray values using the Vidicon camera system at the Image Processing Section (IPS) of the National Cancer Institute as previously described [3]. Twenty-two "landmark" spots were identified in each image using the interactive video display system of the IPS using one of the images (accession number 324.1) of proteins from an AML sample as the "Rgel" and each image was "segmented" into its component spots using the GELLAB program SG2DRV running on the DEC-10 computer at the IPS [4]. The GELLAB program CMPGEL was used to sequentially match the spots in each image with those in the Rgel and to produce a series of gel comparison files. The resulting spot lists ("GSF" files), the gel comparison files ("GCF" files), the landmark file (LMSELA.LM), and the file containing identification data for each image (GELELA.ID) were transcribed to magnetic tape and transferred to the DEC-20 computer at the University of Chicago for further analysis. These files were then used by the GELLAB program CGELP to build a unified data base (JUN27A.PCG) which was subjected to statistical analysis within CGELP. Selected statistical results were verified independently using the MINITAB package on the DEC-20 at Chicago and initial results were visually analyzed using plots created at Chicago with the DISPLA graphics package [3]. After preliminary analyses were complete, the data base and associated raw data output files ("SPS" files) were transcribed to tape, transferred back to the DEC-10 at the IPS and used to create R-map and mosaic images on the video display at the IPS using the MARKGEL and SEERSPOT programs in GELLAB [7].

3 Results

3.1 Data base assembly

Visual inspection of the initial 2-D gel fluorographs of leukemic cell proteins revealed an overall similarity of the patterns when samples from different patients were compared. When two different fluorographs were superimposed, the majority of spots could be identified as present in each image. However, a relatively large number of quantitative differences in relative spot density, and a smaller number of qualitative differences (absence in one gel, presence in another) were noted. We thus elected to begin our analysis with samples from a small number of patients and to seek major differences between the standard classes of leukemia. Our initial work revealed that gels of replicate cell samples pulse-labeled simultaneously showed nearly identical patterns (data not shown). However, it was clear that the spot detection algorithms in SG2DRV, while quite effective for well-defined free-standing spots, may nonetheless show considerable variation in their detection of spots within clusters or streaks, even when analyzing replicate digitizations of a single gel image. We thus elected to digitize a single image from each patient three times and separately segment these. This potentially permitted a meaningful statistical comparison of the three digitizations of one sample with those from a second patient and also will ultimately permit construction of a "C-gel" segmentation file (spot list) statistically summarizing for each sample the data in its three digitizations [8].

The data base reported here consists of the three digitized images of one gel fluorograph from each of 3 patients with AML (one M_1 [325.1-.3 and two M_2 [324.1-.3 and 326.1-.3] by FAB classification), one with ALL (369.1-.3), two with CLL (374.1-.3 and 378.1-.3), and two with HCL (382.1-.3 and 384.1-.3). One image, 378.1, was omitted due to a technical failure in data transmission. The data base was constructed using the sure and possible pairs (SP and PP) to create 678 initial Rspot sets; the unpaired spots were used to create 247 extended Rspot sets and the data base was then extrapolated [8]. The resulting 925 Rspot sets contained 17184 spots (1746 sure pairs, 4111 possible pairs, 3502 ambiguous pairs, 1159 "unresolved" spots, and 6666 extrapolated pairs) in 23 gel images. For statistical analyses the data base was constricted (prefiltered) using the CGELP SET labels command to only the SP and PP spots. For missing class searches (to detect the presence or absence of a spot in one or more class of leukemias) the data base was extended to include "unresolved" spots not paired with the Rgel but paired with each other in the extended Rspot sets (SET labels = PSUX). The spot density values in the data base were then normalized.

For this purpose a normalization set of Rspots was selected by searching the data base for Rspot sets which contained a member from each image in the data base and for which mean absolute density was > 3 (to exclude low density spots likely to represent "noise"). The 44 spots found are displayed in Fig. 1; many are also landmark spots (Fig. 1). The value of each spot in each image in the data base was then expressed as a ratio to the sum of these 44 spots in its image multiplied by 100.

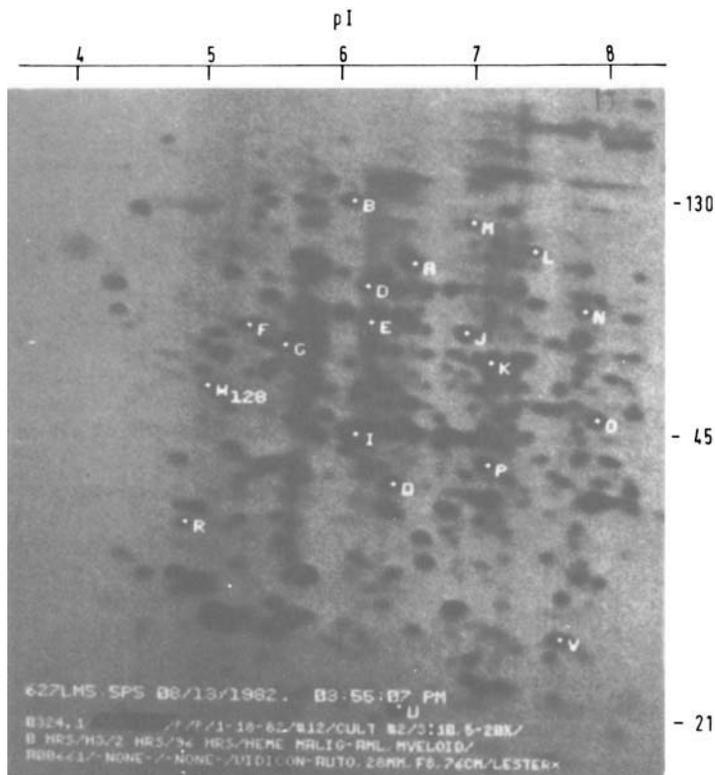


Figure 1. Landmark spots (top) and normalization spots (bottom). The top panel shows the set of 22 landmark spots identified in each image and used by the gel comparison algorithms in CMPGEL. The panel is a photograph of the video display screen at the IPS showing the R-gel (324.1, from an AML patient). An "Rmap" has been inscribed on the gel using the program MARKGEL and the raw data output file "627LMS.SPS" created by CGELP and containing the landmark spot information. MARKGEL has included identifying information at the bottom of the image. Landmark C is missing because of operator error at the time of landmark selection for the Rgel (the point indicated by the operator was > 5 pixels from the centroid of the segmented spot in that location). Landmark H was mispaired in two images with Rspot 128, accounting for its presence in the image. The correct landmark H is Rspot 126. The approximate pI scale is indicated above the figure. The approximate molecular weight in daltons $\times 10^3$ is indicated to the right. The lower panel shows the Rgel on which MARKGEL has inscribed the 44 spots the sum of which in each image was used to normalize the spot density values in that image.

3.2 Quantitative spot differences between types of leukemia

In the analysis of a data base using GELLAB, a number of choices present themselves: 1) mode of normalization; 2) choice of classes to be compared (classes of leukemia in this case) and their definition; 3) choice of statistical procedure (F-text, T-text, Wilcoxon rank sum test); and 4) selection (prefiltering) of Rspot sets to be analyzed by the statistical procedure. For our initial work here we used only

Rspot sets highly likely to contain only correctly paired spots (SP and PP) but we applied no other prefiltering restriction (such as a lower bound on density or area to reduce "noise"). Since the response of film to radiation is non-linear and our spot measurements are made in terms of density rather than being converted to absolute counts per minute, we currently feel that a non-parametric statistical procedure such as the Wilcoxon rank sum (WRS) test is most appropriate for our data. Thus, using the data base after normalization against

the initial set of 44 normalization spots, we performed a statistical search comparing 9 AML images from 3 patients with 5 CLL images from 2 patients using the Wilcoxon rank sum to find Rspot sets in which the density values for class 1 differed from those of class 3 with a probability less than or equal to 0.01. Forty-one Rspot sets were found and their numbers were stored in a search results list (SRL 4) within GELLAB. They are displayed as R maps (marked images) in an AML image (top) and a CLL image (bottom) in Fig. 2. A similar comparison of AML vs. HCL revealed 39 Rspot sets differing with a $p \leq 0.01$ (SRL # 6). These are displayed in Fig. 3. The intersection of these two sets included 14 Rspots

(SRL # 7 displayed in Fig. 4) which are thus those spots that differ quantitatively between AML and both CLL and HCL. Our current understanding of the biological relation between leukemias would lead us to expect such a result - some proteins will be differentially expressed in both lymphoid leukemias (CLL and HCL) when compared with AML. In view of the extremely limited size of this initial data base, we have not attempted to compare CLL with HCL. Clearly, this set of 14 Rspots may be incomplete and may also include inappropriate Rspots since inaccuracies in spot detection and pairing will influence its composition. Any such result requires visual confirmation and correction (*vide infra*).

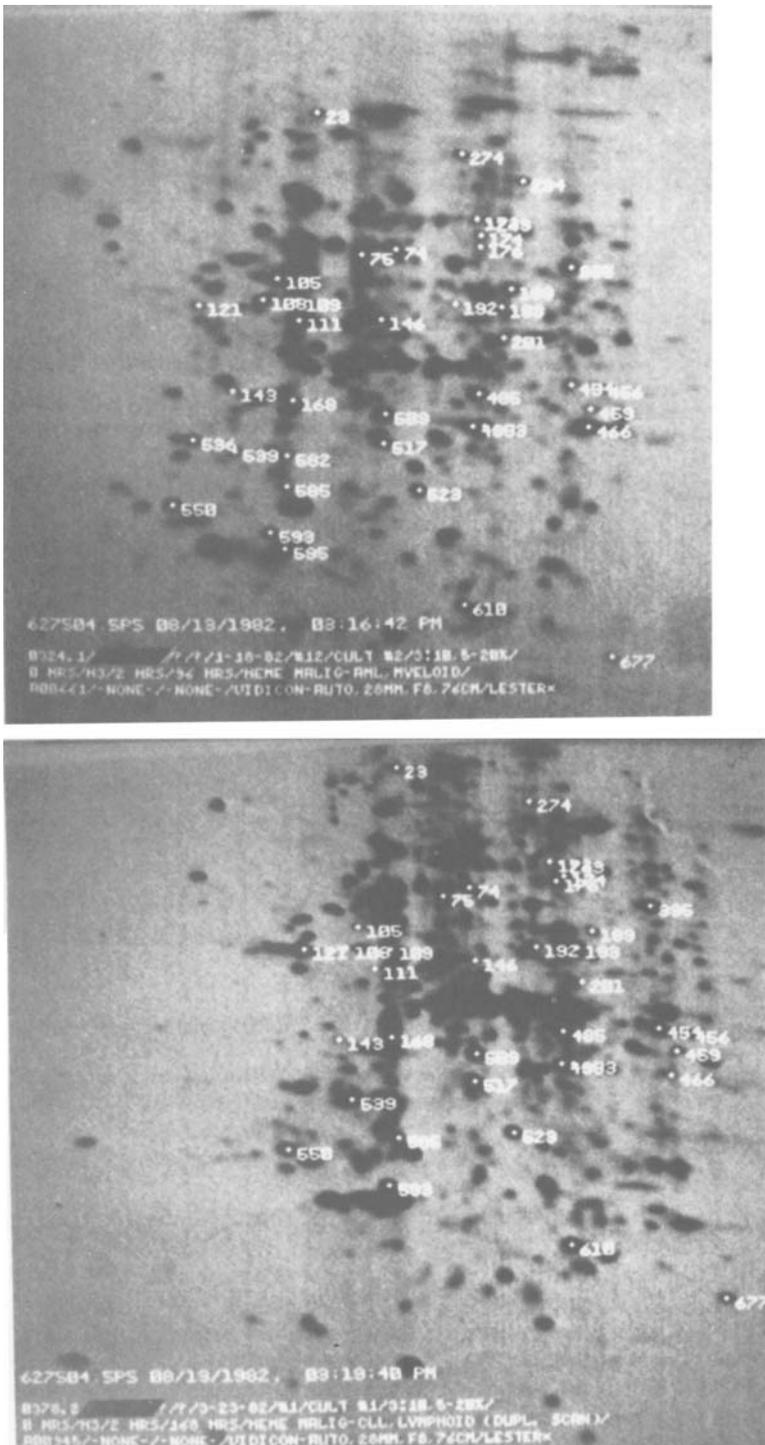


Figure 2. AML vs. CLL, WRS $p \leq 0.01$. Rmaps showing spots (Rspot sets) found by a WRS test to vary by $p \leq 0.01$ when AML and CLL samples were compared. The top panel shows the Rgel, an AML sample, on which the spots found by the statistical search are marked. The bottom panel shows the same set of spots marked in an image from a CLL sample (gel image 378.2). The accuracy of spot pairing can thus be judged.

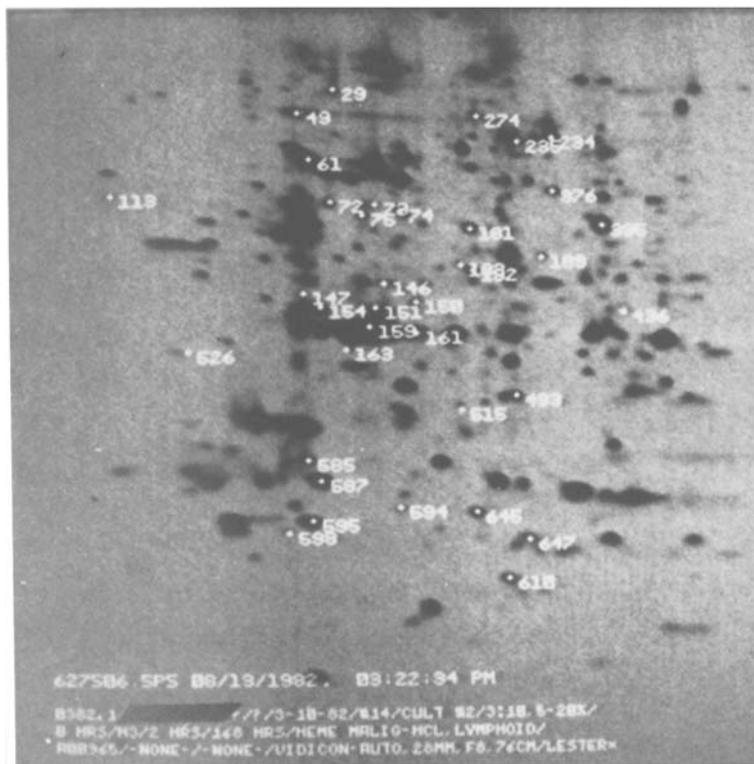
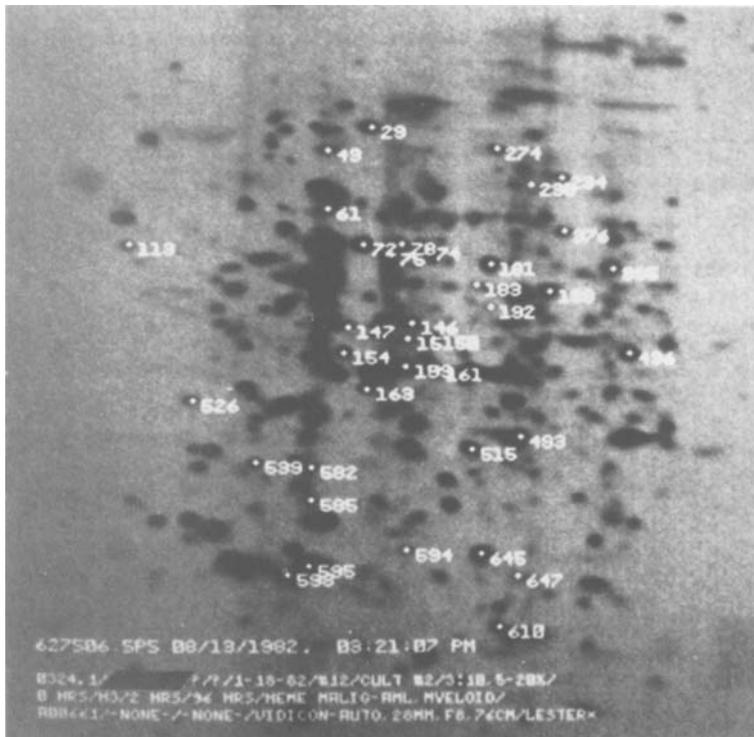


Figure 3. AML vs. HCL, WRS $p \leq 0.01$. *Rmaps* showing the set of spots varying by $p < 0.01$ when a WRS test was used to compare AML and HCL samples. The top panel shows the search result list inscribed on the *Rgel* (an AML sample) and the bottom shows the same list displayed on an HCL sample (gel image 382.1).

We next re-defined the classes of gels to be considered and compared the 9 AML images with all 14 of the lymphoid images (from 1 ALL, 2 CLL, and 1 HCL patients). A WRS statistical search revealed 62 and a T-test gave 23 Rspot sets differing between myeloid and lymphoid images with a $p \leq 0.01$. The 20 spots in the intersection of these sets are shown in Fig. 5 (SRL \neq 17). Nine of them were found previously in the set of 14 spots which differ in both CLL and HCL in comparison with AML (Fig. 4). This analysis has

been supplemented by re-normalizing the data base using a set of only 7 spots (chosen from the initial set of 44 normalization spots by inspecting the density values of each of the 44 and choosing Rspot sets which appeared to be optimally defined). A repeat of the myeloid vs. lymphoid T-test at $p \leq 0.01$ found 24 Rspot sets of which 19 were present in the same search using the previous normalization. The data base has also been normalized using a least squares method [9] and the AML vs. CLL and AML vs. HCL analysis has been

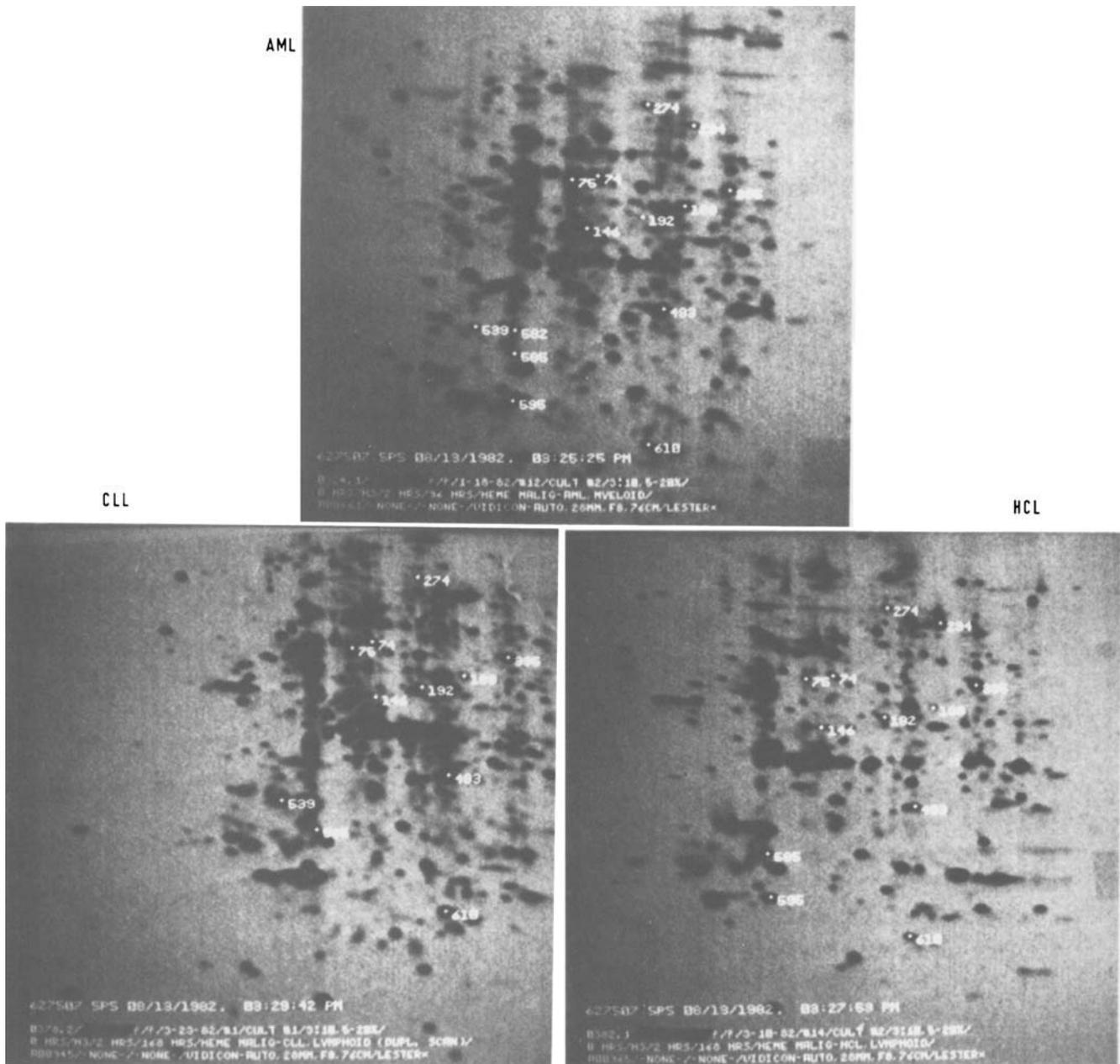


Figure 4. The intersection of the sets displayed in Fig. 2 and 3. The spots found in common in both the AML vs. CLL and the AML vs. HCL. WRS search lists are displayed in an AML image (324.1, top panel), a CLL image (378.2, lower left panel) and an HCL image (382.1, lower right panel).

repeated. The results appear to be substantially similar although Rspot sets of borderline significance may or may not be found by a particular statistical search depending on the normalization used.

While the finding of substantially similar groups of spots using a variety of statistical procedures and normalization methods provides a measure of confidence in these results, visual verification is essential. This is particularly true in the developmental phases of a 2-D gel analysis system such as GELLAB. Fortunately, it is still feasible with the relatively small number of gels and Rspot sets of interest in this initial data base. Graphic plots of each gel were made on the Calcomp plotter at the University of Chicago using the DIS-

PLA graphics package and used to verify that the spots found by statistical searches did indeed differ between myeloid and lymphoid samples. Furthermore, Rmaps (marked images) and mosaic images were generated at the Image Processing Section, NCI, photographed, and used for visual verification in addition to the 8 original gel fluorographs comprising this data base. Fig. 6 shows the current set of visually checked Rspots which appear to be candidates for useful quantitative markers to distinguish myeloid from lymphoid leukemias. Fig. 7 shows a sample mosaic image in which a particular Rspot of interest (Rspot 234) is displayed in each image in the data base, thus permitting visual verification of its low density in myeloid as compared to lymphoid samples.

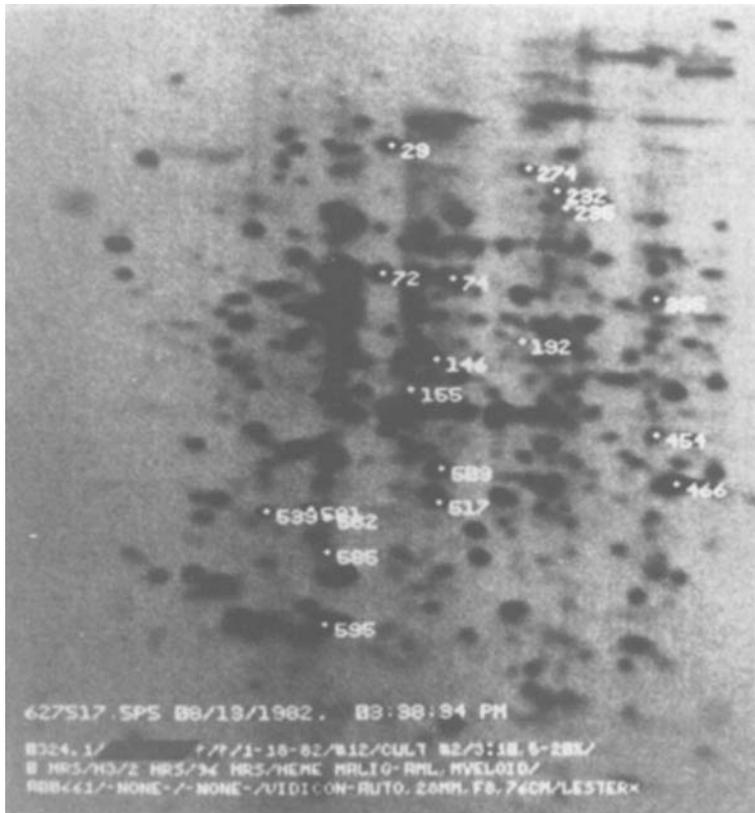


Figure 5. Myeloid vs. lymphoid, $p < 0.01$ by WRS and t-test. The set of spots found in common by both the WRS and the t-test as having a $p < 0.01$ when all myeloid images were compared with all lymphoid images. The spots are displayed in the Rgel.

3.3 Qualitative differences in 2-D gel images from differing types of leukemia

GELLAB is equipped with the capacity to identify Rspot sets in which a given class of samples is entirely absent using its "missing class search" facility. Obviously, the meaning of such a finding is dependent on the sensitivity of the electrophoretic technique generating the image and the spot detection algorithms. By prefiltering the data base so as to consider only SP and PP spots and Rspot sets with a least 5 of the 9 myeloid images present and then performing a missing class search comparing AML and CLL, we found 16 candidate Rspot sets. A similar comparison of AML and HCL samples found 19 Rspot sets, 7 of which had also been found in the AML vs. CLL missing class search. These necessarily represent proteins present in at least two of the myeloid but not in the lymphoid leukemia cells.

The converse analysis was also performed by restricting the data base to the extended Rspot sets (Set Label = UEXX), and prefiltering to require a relatively robust density (mean normalized density 0.4) and at least 6 samples in either set present per Rspot set. A missing class search with the two classes for comparison defined as all myeloid vs. all lymphoid images revealed 23 Rspot sets. These represent candidate proteins for markers of at least some lymphoid leukemias since they are not found in any of the 3 myeloid samples. Six of these spots have been visually verified as qualitative changes (Fig. 8) by visual analysis of Rmaps, mosaics, and the original gel fluorographs of each gel in the data base. Fig. 9 is a mosaic image of one of these spots (Rspot 803) showing its absence in myeloid and presence in lymphoid samples. A number of additional polymorphisms are apparent in this region of the gels.

3.4 Global measures of similarity between 2-D gel images

The analysis of qualitative and quantitative differences in individual spots and sets of spots which we have just reported provides a useful strategy for identifying marker proteins of potential diagnostic utility. But it is clear that examination of a single marker can only create a dichotomous separation of leukemias and individual markers may give conflicting results. Thus some form of summary statistic expressing the overall relatedness of 2-D gel images is needed. As a first approximation to such a statistic, we have used the correlation coefficient of the SP and PP of each possible pair of gels in the data base using the GELLAB "tabulate" function. Table 1 shows this matrix of correlations of replicate digitizations. Replicate digitizations showed correlation coefficients in the range of 0.89-0.99, as expected. AML samples

Table 1. Correlation coefficients of sure and possible pairs^{a)}

	AML	AML	AML	ALL	CLL	CLL	HCL	HCL
Gel #	324	325	326	369	374	378	382	384
AML 324	0.99	0.77	0.65	0.63	0.45	0.56	0.51	0.65
AML 325		0.99	0.54	0.55	0.47	0.46	0.46	0.63
AML 326			0.89	0.42	0.32	0.40	0.41	0.50
ALL 369				0.93	0.38	0.48	0.39	0.49
CLL 374					0.92	0.47	0.48	0.50
CLL 378						0.90	0.59	0.59
HCL 382							0.91	0.62
HCL 384								0.95

a) Each value represents the mean correlation coefficient when corresponding gel images, in their various digitizations, were compared. The number of pairs used for correlation ranged between 104 and 349.

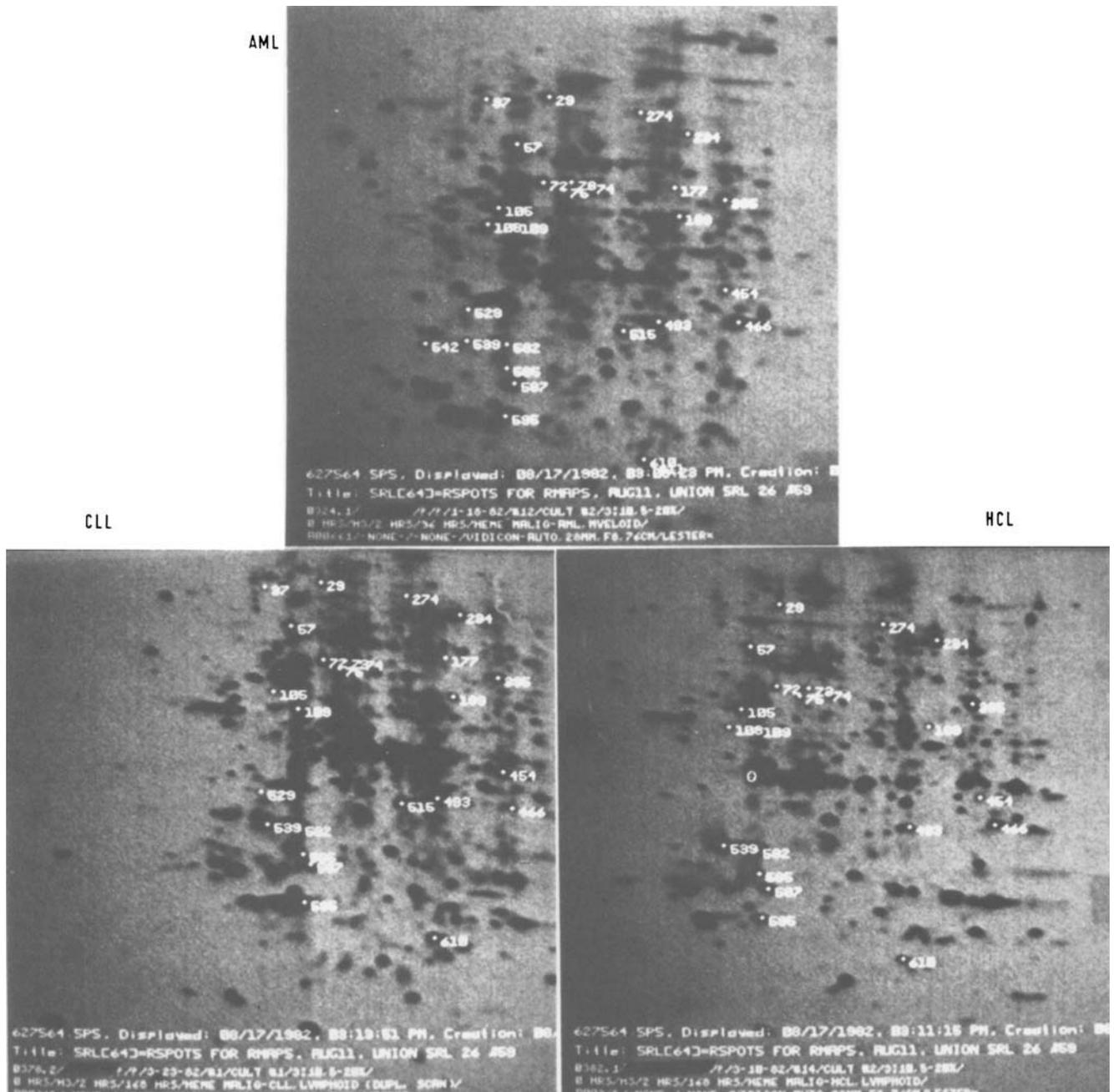


Figure 6. Candidate spots for quantitative markers serving to distinguish AML from CLL or HCL. Each of the images shows the list of spots detected by the preceding statistical analysis of the data base and subsequently verified by visual checking of the original fluorographs of each of the AML, CLL and HCL images. These represent potential markers to be evaluated in larger data bases in the future.

correlated with each other in the range of 0.54–0.77 and were similar to the correlations of ALL with AML. CLL was least well correlated with AML (0.32–0.56) and HCL was intermediate (0.41–0.65). This might be expected on biological grounds since HCL cells generally share B lymphocyte and monocyte/macrophage properties. Nearly identical results were obtained when the correlation coefficient matrix was generated using the data base after least squares normalization and with the inclusion of extended Rspot sets (data not shown). The data is far too scant for meaningful biological conclusions, but this or similar methods may have widespread utility.

4 Discussion

Perhaps the most important conclusion of this work is that a comparison of 2-D gel patterns of cellular proteins synthesized by leukemic cells shows both major similarities and great differences when different leukemias are compared. The similarities permit an accurate comparison of samples; the differences may potentially be used to create new and more meaningful classifications of leukemias. While the major currently accepted classes (AML, ALL, CLL, HCL, *etc.*) are unlikely to change, the heterogeneity within these classes

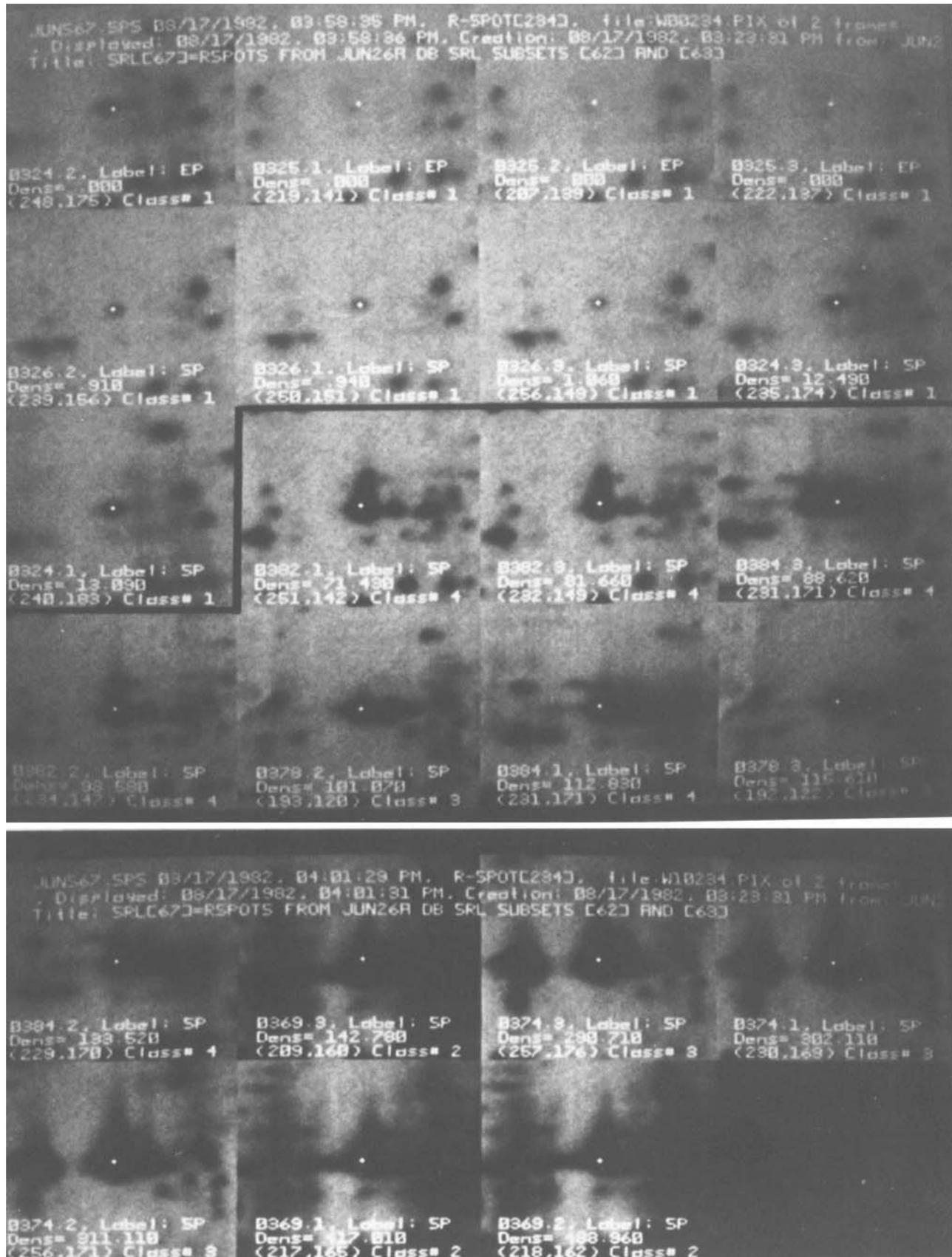


Figure 7. Mosaic image of Rspot 234. Each section of the figure shows Rspot 234 and the surrounding gel region from one of the 23 images in the data base. Rspot 234 is a spot which is relatively small in AML but large in lymphoid leukemias. The upper 9 segments (above the heavy line) show the myeloid samples; the lymphoid samples are below. Additional local polymorphism is apparent. Sixteen segments were displayed in a single mosaic image on the video display; two video display images were photographed and combined in this figure.

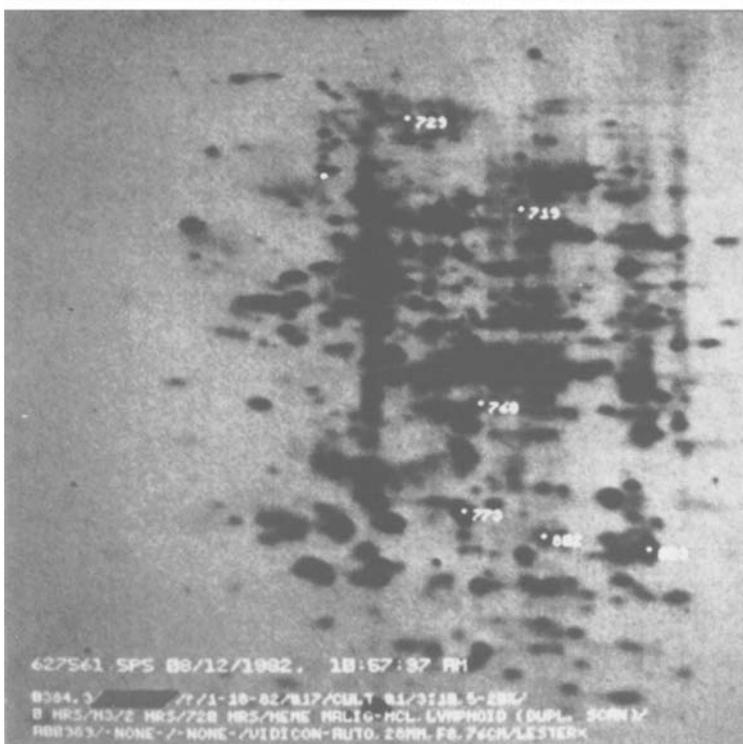
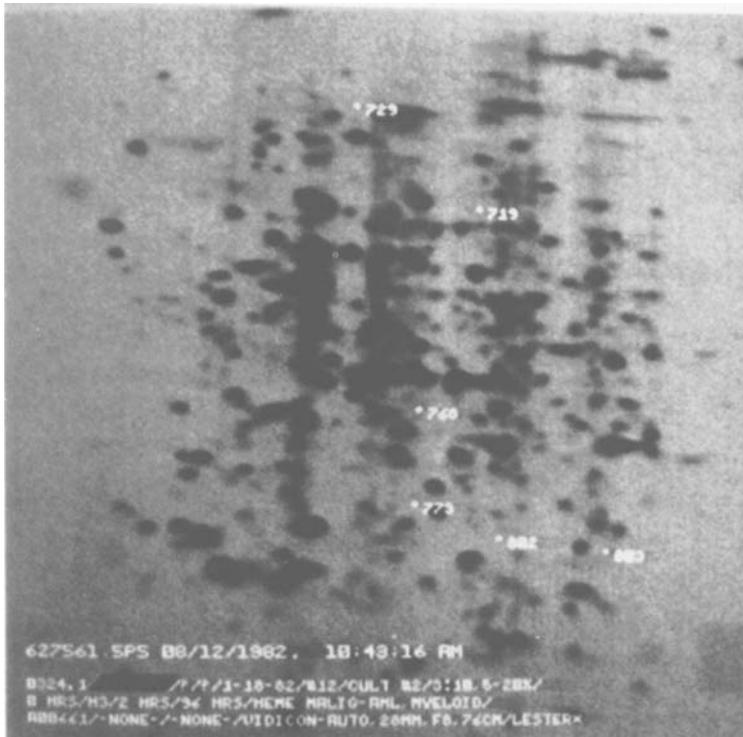


Figure 8. Selected qualitative spot differences between myeloid and lymphoid samples. Six Rspot sets detected by missing class searches as present in some or all lymphoid samples but absent in all myeloid samples are displayed on an AML image (324.1, top) and an HCL image (384.3, bottom). Visual checking of the original fluorographs in the data base confirmed their presence in lymphoid and absence in myeloid samples.

promises more useful subclassifications. Indeed the heterogeneity of patterns seen on 2-D gels seems quite appropriate when viewed in the context of the clinical and biological heterogeneity seen within our current classes of leukemia.

Since these 2-D images implicitly contain information about the major aspects of genetic expression occurring in these cells, they should provide a far more complete and precise description of their state of differentiation than any other

form of marker analysis. Information from such studies as cell surface markers, enzyme histochemistries and reactivity with monoclonal antibodies is all largely implicit in the 2-D gels. Additional information regarding cell cycle kinetic status may also be inferred. Indeed, Rspots 75 and 105, which are quantitatively relatively reduced in the CLL and HCL samples, are proteins whose relative synthetic rates we have previously shown to be related to growth activation in normal lymphocytes [2]. We would expect these proteins to

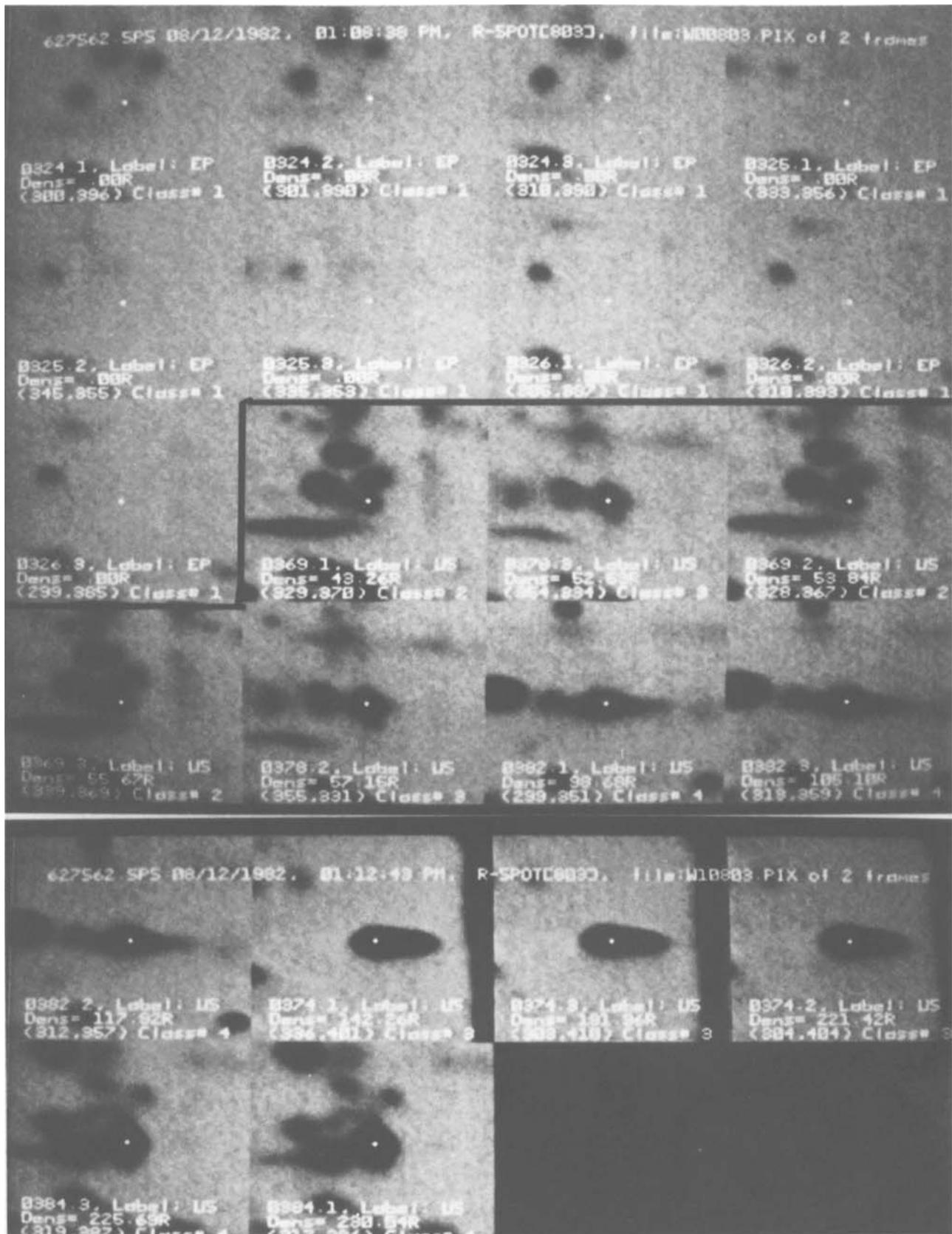


Figure 9. Mosaic image of Rspot 803, a spot present in lymphoid but absent in myeloid samples. The upper 9 segments are from myeloid images, the lower segments from lymphoid.

show lower relative synthetic rates in CLL and HCL cells than in AML or ALL cells since the growth rates of cells from the chronic leukemias are generally lower than those from the acute leukemias.

The relative role of qualitative as opposed to quantitative differences in protein composition in the establishment of a differentiated phenotype is unknown. Ideally, both should be analyzed. Clearly, the spots which have been found to differ significantly between types of leukemic samples in this data base can only be viewed as candidates for meaningful markers in view of the small number of samples. Nonetheless, some of them will likely be of real utility. Simple visual comparison of the relative densities of Rspots 234 and 274 has permitted the distinction of myeloid from lymphoid leukemias in a larger series of 35 leukemic samples (manuscript in preparation). Computerized analysis of a larger data base will clearly be required and, ultimately, careful correlation of the gel patterns with clinical features such as organ involvement, response to therapy and survival will be needed. The discovery of additional protein markers distinguishing the major classes of leukemias may be of therapeutic as well as diagnostic importance in view of the potential for the development of monoclonal antibodies against these proteins.

A number of additional hypotheses may be tested in future work with this system: 1) Clinical experience suggests that the separation of leukemias into discrete diagnostic groups, while useful, is artificial. Patients whose disease seems to bridge one or more diagnostic categories are not uncommon. Can we use 2-D gels to delineate a multi-dimensioned spectrum of leukemias into which each individual's disease may be placed? The use of summary statistics, such as correlation coefficients or cluster analysis may permit this. It may be best to utilize one or more subsets of spots for this purpose since some proteins may vary between samples for reasons having little to do with clinically important factors. 2) Can we correlate degrees of malignancy and de-differentiation with aspects of the 2-D gel patterns? Do the more malignant leukemias express a greater number of gene products as a manifestation of genetic derepression? 3) Can we follow clonal evolution in these malignancies, as is now possible

with karyotypic analysis, using 2-D gel protein patterns? 4) Can we find sets of proteins whose genetic expression may show common regulatory mechanisms as evidenced by an apparent linkage in their relative synthetic rates? How does the composition of these sets differ in various cell types?

These and a wealth of other clinical and biological questions now appear to be increasingly approachable. We believe that the computerized analysis with GELLAB of this initial small data base of 2-D gels of leukemic cell proteins provides evidence for the likely clinical utility of this approach.

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