Quantitative Tracing of mRNAs for T- and B-Lymphocyte Receptor Genes in Individual Cells by In Situ Hybridization With Fluorochrome-Labeled Gene Probes. I. Expression in Malignancies Carrying B-Lineage Associated Antigens

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Acute and chronic lymphatic leukemias were investigated on the single-cell level for the activity of genes coding for the IgM heavy chain and the α and β chains of the T-cell antigen receptor (TCR). We used a new method for preparing highly fluorochrome-labeled gene probes for in situ hybridization, which allowed rapid and quantitative detection of mRNA at the individual cell level. Leukemic cell populations classified as belonging to the B lineage according to their surface antigenic patterns revealed increasing expression of mRNA for the IgM heavy chain (μ mRNA) in a maturation-dependent fashion, which was not correlated to rearrangement of the immunoglobulin μ chain gene—only 66% of the leukemias with rearranged μ gene also transcribed it. TCR mRNA was detected in B-antigen positive leukemic cells. High levels of both TCR and μ mRNA expression in all cells of some of these leukemias allowed the conclusion that these cells simultaneously transcribed the genes for T and B cell antigen receptors. TCR mRNA was also found in what are considered relatively mature B leukemias, lineage cross-over on the mRNA level being observed at a frequency of 23% (five of 22 cases), comparable with that of "inappropriate" receptor gene rearrangement. The quantitation of mRNA with fluorochrome-labeled gene probes in situ may allow determining the degree of gene activation in individual antigenically defined cells and may thus contribute a new tool for characterization of normal and malignant cells.

TRANSCRIPTIONAL PROCESSES play a central role in cell proliferation and differentiation. Quantification of the transcription product, the mRNA, may contribute important information concerning maturation of normal and malignant cells. Biochemical methods routinely used for investigation of mRNA, such as blot hybridization, require large and uniform populations for analysis and provide information only for whole populations, not for individual cells.[1] In situ hybridization, in contrast, is applicable to relatively small cell populations[2] and can also yield results in heterogeneous populations. Moreover, it allows for identification and location of the mRNA of interest in individual cells. Conventional methods using radioactively labeled gene probes are hazardous to handle and time consuming, require special precautions, and do not provide quantitative results.

Fluorochrome-labeled gene probes are easy to trace by fluorescence microscopy and their use offers the possibility of quantifying the results (Pachmann et al, submitted).[3] We applied this method to leukemic cell populations in order to learn more about the maturation of normal and leukemic cell populations along the B cell and the T cell axis. In combination with cell surface analysis using monoclonal antibodies proven useful in helping classify normal and neoplastic lymphoid cells at different stages of maturation,[4] we studied the two structures that have up to now been assayed to distinguish B cells from T cells: namely immunoglobulin M and the T-cell antigen receptor. The transcriptional activity of the genes coding for the IgM heavy chain μ and the TCR α and β chains were quantitatively measured by hybridizing with fluorochrome-labeled DNA probes. Parallel assay of the cell surface antigens CD3, CD5, CD10, CD19, CD20, CD22, CD24, and immunoglobulins with conventional immunofluorescent methods allowed correlation of the specific mRNAs to the stage of B-cell differentiation.

MATERIALS AND METHODS

Cytological preparations. Cells from peripheral blood of patients were isolated by Ficoll Isopaque separation and were washed twice in phosphate buffered saline solution (PBS) pH 7.4, and 10^6 cells sedimented in 10 μL of PBS. The cell membrane was made porous by suspending the cells in 500 μL of hypotonic medium (0.9% trisodium citrate), and two minutes thereafter, fixed by adding 500 μL of acetone. After two hours fixation, the cells were cytocentrifuged directly onto slides at 520 rpm at a density allowing at least three cell diameters of free space between the individual cells.

Labeling of DNA and in situ hybridization. DNA coding for the immunoglobulin μ constant fragment (80 base pairs of domain C3 and all of domain C4 plus intervening sequences) was kindly provided by Dr M. Pech (Institute for Physiological Chemistry, University of Munich) and produced according to the sequence published by Rabbitts et al.[5] The 920 nucleotide fragment was used for hybridization.

To mark mRNA of the TCR α chain, we used cDNA coding for the constant region of the TCR α chain, derived from poly(A)+ RNA of the Jurkat T-cell line.[6] For mRNA of the TCR β chain we used cDNA specific for the constant regions of the TCR β chain derived from poly(A)+ RNA of the MOLT-3 T cell line.[7] Salmon sperm DNA was used as a negative control. Fluorescein isothiocyanate (FITC) was obtained from Fluka (Buchs, Switzerland).

As has been previously described (Pachmann et al, submitted), FITC was conjugated to the synthetic polypeptide polyethylenimine (PEI) (Serva, Heidelberg, FRG) at an FITC/PEI ratio of 9 μg/5 mg. The degree of labeling was measured by absorbance at 280 nm for the peptide and 490 nm for fluorescein.

A DNA probe was denatured and then coupled to the fluoresceinated PEI using 0.008% glutaraldehyde. Excess binding capacity of

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the glutaraldehyde was either saturated by adding 5 μL of 1.5 mol/L L-lysine or 5 μL of 5% bovine serum albumin (BSA). Hybridization reactions were carried out with a modified method of Fournier et al.[8] The probe was diluted with hybridization buffer consisting of a 100 μL mixture of 10 mmol/L Tris HCl (Sigma, Deisenhofen, FRG) pH 7.5, 1 mmol/L EDTA (Sigma), 600 mmol/L NaCl, 0.02% Ficoll (Sigma), and 0.02% polyvinylpyrollidone (Sigma), and 10 μL was added to each slide. Reactions were sealed under a coverslip using a vulcanizing glue and the slides incubated for 24 to 48 hours in a humidified atmosphere at 37°C. The slides were then unsealed in 1 × SSC buffer and washed once in the hybridization buffer containing 50% deionized formamide and once in the hybridization buffer without formamide. They were then washed again in 1 × SSC, covered with one drop of glycerine, and the fluorescence measured. Measurements were performed on a Leitz Orthoplan microscope photometer MPV 2 equipped with a Ploem optique for fluorescein and rhodamine illumination. The fluorescence intensity of each cell was measured, transferred to a Commodore CM 8032, and stored on a floppy disk. Relative intensity values were standardized with an external fluorescence standard. Background measurements were performed on cell-free spaces adjacent to the cells and were subtracted automatically from the total cellular fluorescence value. Corrected net values were plotted as a frequency distribution diagram.

Surface antigen screening tests. The following antibodies were used (the clusters of differentiation according to the WHO criteria given in parentheses): T28 (CD3), Lyt2/Leu1 (CD5), Vil A1 (CD10), HD37 (CD19), B1 (CD20), HD6/39 (CD22), BA-1 (CD24), anti-polyvalent Ig and anti-IgM. Cytoplasmic IgM was also quantitated in a few cases.

Indirect immunofluorescence staining was performed with standard methods using the monoclonal antibodies as the first reagent and affinity-purified fluoresceinated goat anti-mouse globulin reagents (Tago Hamburg, FRG). Before use the reagents were ultracentrifuged and checked for nonspecific binding using the K562 cell line. Evaluation was performed using a Leitz microscope with epillumination.

Northern and Southern blotting was performed using established standard methods.[1,5]

RESULTS

Forty-one lymphatic neoplasias were studied. The clinical classifications were as follows: 24 acute lymphatic leukemias (ALL), eight chronic lymphatic leukemias (CLL), five non-Hodgkin's lymphomas (NHL), one acute undifferentiated leukemia (AUL), two prolymphocytic leukemias (PLL), and one plasmocytoma. Peripheral blood lymphocytes from healthy individuals and from the cell lines MOLT-4, Jurkat, and Marshak Rothstein (an IgM producing hybridoma) served as controls.

Cells were hybridized with either μ, TCRα, or TCRβ DNA probe. One hundred cells of each population were measured and the fluorescence intensities recorded and compiled as fluorescence intensity histograms. Typical histograms showing the distribution of fluorescence intensities in in situ hybridization for μ, TCRα, and TCRβ mRNA are given in Figs 1, 2, and 3. They display a representative sample of histograms showing the frequency (% cells) found for each corresponding fluorescence intensity, which gives a measure of the gene activity in each cell population. Leukemic cell populations were found with varying degrees of transcriptional activity, from very high to zero.

The histograms shown are selected from different B-maturation stages. The numbers of the leukemias correspond to those given in Table 1. Most of the populations measured resulted in a Gaussian distribution of values, but some cases showed a second peak, indicative of two separate populations. Thus the cells of an ALL (no. 11, Fig 1) carrying only CD10 and CD24 showed only a small population of μ mRNA expressing cells. This case and comparable ones were termed ± in Table 1. However, also a small population with TCRβ mRNA and even slight TCRα mRNA expression were observed.

Another ALL (no. 21, Fig 2), which expressed CD22 and

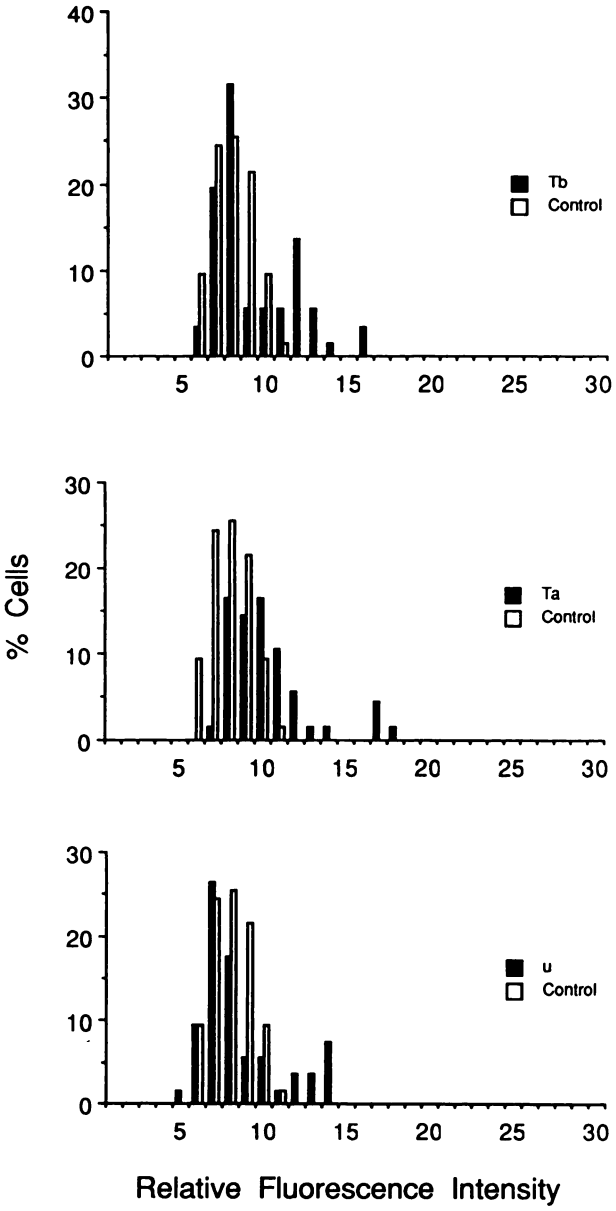

Fig 1. Frequency distribution of fluorescence intensities of 100 individually measured cells in a pre-B cell leukemia (patient no. 11 of Table 1) hybridized with immunoglobulin μ, TCR α, and TCR β DNA probes. Control was salmon sperm DNA.

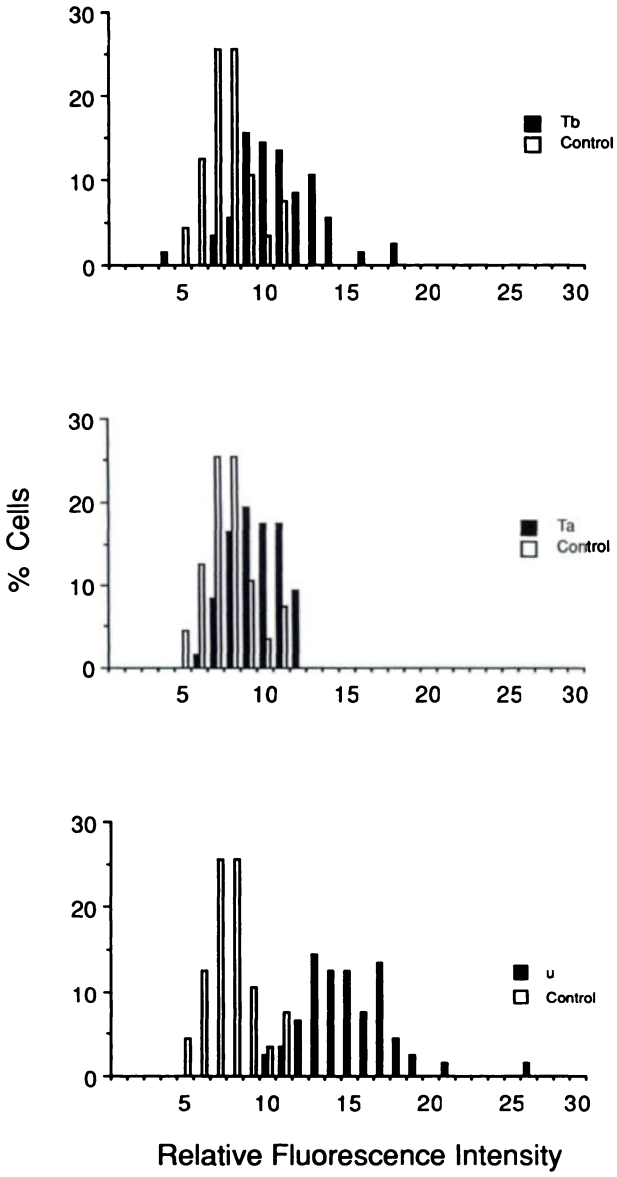

Fig 2. Frequency distribution of fluorescence intensities of 100 individually measured cells in a cytoplasmic μ chain positive pre-B cell leukemia (patient no. 21 of Table 1) hybridized with immunoglobulin μ, TCR α, and TCR β DNA probes. Control was salmon sperm DNA.

CD20 and contained cytoplasmic μ chains,[9] displayed a high level of μ mRNA. TCRβ mRNA showed a main peak in the range of the negative control (background binding always being assayed with an unrelated fluorochrome labeled probe) with a slope to the right, indicative of a small population not sufficiently distinguishable from the negative population by intensity. Such cases were also classified as ±. Slight TCRα activity was observed in the whole population, as seen from the shift of the entire histogram to the right. A NHL (no. 33, Fig 3), on the other hand, expressed high levels of μ, TCRβ, and TCRα mRNAs.

Accumulated results of all leukemic populations are shown in Table 1. CD3 was used to measure the amount of T cells in the populations and never exceeded 10% in any of the 17 cases studied. Cell surface markers are listed from CD19 to Ig in the currently postulated order of appearance during B cell maturation. In addition, the cytoplasmic IgM levels as determined by quantitative immunofluorimetry are shown for four leukemias. Relative values of the amount of μ mRNA, as determined from the fluorescence intensity histograms, are given as ± to +++ with reference to a control stained with an unrelated DNA probe.

In the acute leukemias the fluorescence intensities increase with increasing expression of B maturation markers before surface IgM expression. The level of μ mRNA expression was highest shortly before and at the stage where IgM appeared on the cell surface and correlated well with cytoplasmic IgM content in the four cases tested.

The chronic lymphatic diseases did not represent a homogeneous group. Among those carrying only IgM on the cell surface, cases expressing low and high μ mRNA were observed. An IgM-expressing CLL (no. 29) showed high μ mRNA expression, but neither TCRα nor TCRβ mRNA.

Remarkable was the expression of IgM on the surface of CLLs with low levels or no μ mRNA found (cases no. 25, 26, 27). We have tentatively grouped them in an order with increasing μ mRNA content. During further maturation the amount of μ mRNA diminished, being absent in those CLLs that had completed the switch from IgM to another immunoglobulin heavy chain class. For example, in a CLL (no. 38) where most cells had completed the class switch (>90% Ig^+, 20% IgM^+), μ mRNA was no longer expressed, but we detected TCRα mRNA in these cells.

Both TCRα and TCRβ mRNA were detected in some leukemias of apparent B-cell lineage, even in those classified to later maturation stages. Parallel, even strong activation of both the μ and the T cell antigen receptor genes was observed in two NHLs (no. 31 and 33) investigated. Both of these NHLs had negligible amounts of surface CD3 not correlated with TCR mRNA expression.

The μ gene was rearranged in twelve of twelve B-determined leukemias investigated irrespective of the subsequent transcription. That is, in four cases the gene was rearranged without expression and in eight cases rearrangement was accompanied by μ mRNA expression. Northern blots were made to verify the results obtained by in situ hybridization (Fig 4).

DISCUSSION

Initially enthusiastic reports on the specificity of antigen receptor gene rearrangement as a definite marker for classification of acute leukemias have subsequently been challenged by the demonstration of a relatively frequent incidence of cross-lineage rearrangement of the immunoglobulin heavy chain gene in nonlymphocytic leukemias[10-15] and in T cell leukemias.[15-21] Thus these investigations do not allow for unequivocal lineage affiliation.

In order to determine whether the transcription of receptor genes is a more lineage-specific event than rearrangement, we have determined the amount of antigen receptor mRNAs in leukemias corresponding to different stages of B-cell maturation.

We assayed the mRNA by in situ hybridization with fluorochrome-labeled probes, a method that allows more rapid and exact discrimination between background and specific signals than radioactively labeled probes,[3] especially in the low intensity range with a lower sensitivity limit of 50 molecules of bound DNA probe. Even minority populations of cells can be detected, since for a given total amount of mRNA, one can distinguish whether all cells express low levels or only a fraction of the cells expresses high levels.

The fact that most of the leukemic populations showed a Gaussian distribution of values is an indicator of their monoclonality. The leukemias with insufficient separation of a positive population or those exhibiting more than one peak require further investigation. Work is in progress in order to identify by double labeling with surface markers and gene probes the cell population that expresses the receptor mRNA.

In a previous report[9] we already demonstrated that quantitation by in situ hybridization allowed detection of a direct correlation between the mRNA coding for the μ heavy chain and the intracellular IgM content in leukemias representing the early stages of B-cell maturation expressing the cALL antigen. Some of these leukemias were incorporated in the present work (cases 10, 14, 20, and 24). The amount of mRNA for a particular receptor molecule is a measure of its turnover. Therefore a correlation between cytoplasmic IgM and μ mRNA can be expected, but such a correlation does not necessarily exist between differentiation markers expressed on the cell surface and μ mRNA. Nevertheless, in the present report we could show that for the 24 acute

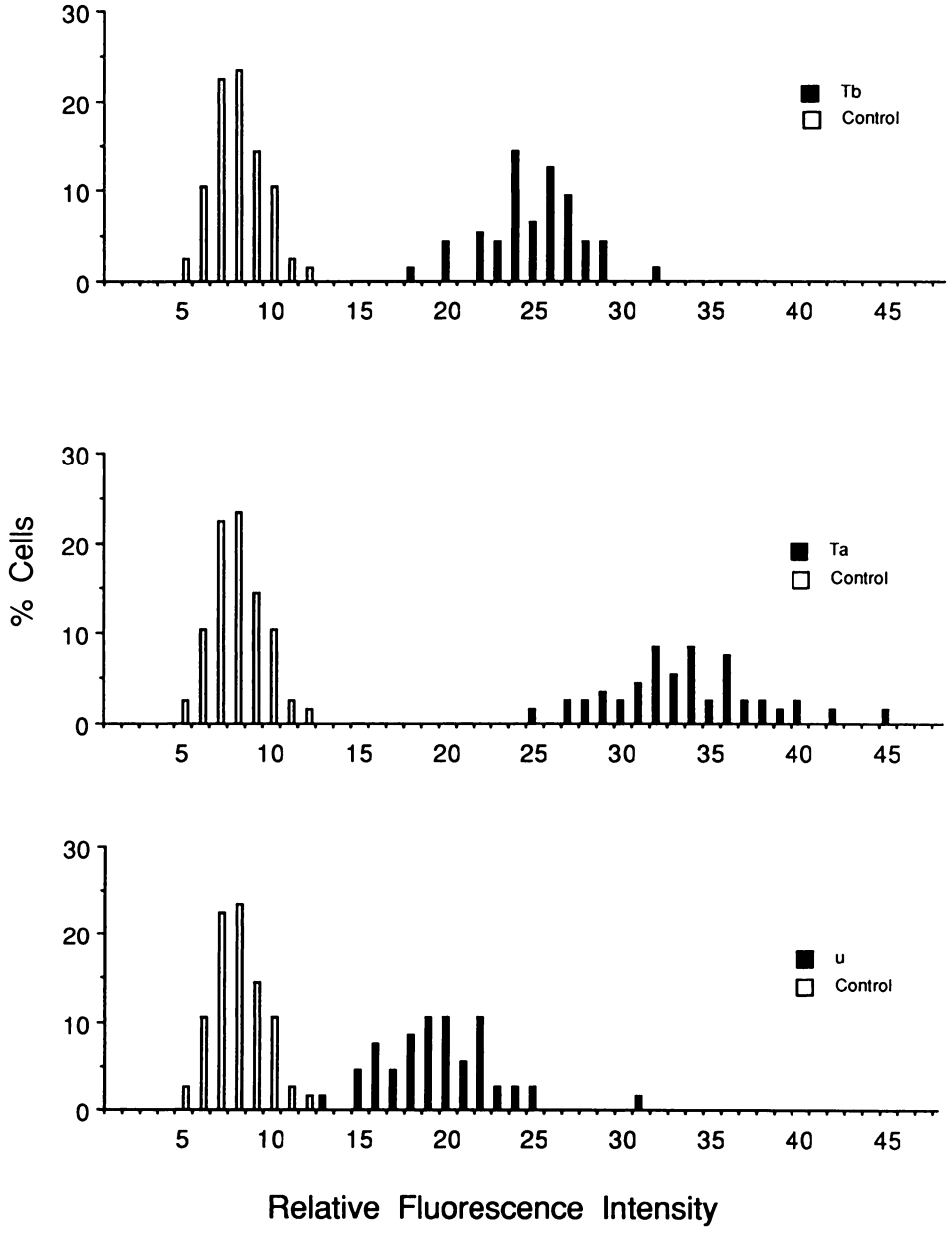

Fig 3. Frequency distribution of fluorescence intensities of 100 individually measured cells in a leukemia carrying IgM and another heavy chain class on the cell surface (patient no. 33 of Table 1) hybridized with immunoglobulin μ, TCR α, and TCR β DNA probes. Control was salmon sperm DNA.

Table 1. Correlation of IgM Gene Expression to the Expression of B-Cell Differentiation Markers and TCR Gene Expression in B-Determined Leukemic Cell Populations

Patient No.	Surface Markers											mRNA				Diagnosis
	CD3	CD19	CD10	CD24	CD22	CD20	E_m	IgM	CD5	Ig	cIgM	μ	TcR_α	TcR_β	Rearr. μ	
1		——	o			o		o	o	o		–				ALL
2		——	o			o		o		o		–				AUL
3	o	——	o			---		o	---	o		+/–	+/–			ALL
4		————						o	---	o		–				ALL
5		————						o	o	o		+/–				ALL
6	o		————			---		o	o	o		–		–	+	ALL
7			o	——				o	o	o		+/–			+	ALL
8	o	——	o	——		---		o	o	o		–	+/–			ALL
9		——	o	——		---		o	o	o		–				ALL
10		————						o	---	o	+/–	+/–				ALL
11	o		————					o	o	o		+/–	+/–	+/–	+	ALL
12	o	————						o	o	o		+		++	+	ALL
13		————			——			o	o	o		+				ALL
14		————				o					+/–	+/–			+	ALL
15		————				---		o	o	o		+/–				ALL
16	o		o	——		——						+/–		+	+	ALL
17		——		o		——		——	o	——		–			+	ALL
18		————				——	o	o	o	o		+/–				ALL
19		————			——			o	o	o		+				ALL
20		————				——		o		o	+	++				ALL
21		————			——			o		o		++	+/–	+		ALL
22		————				---		——				++				ALL
23		————				——		——				++				ALL
24		————			——			——			++	+++			+	ALL
25	o		o	——			————			o		–	+	+/–	+	CLL
26	o			——			---	————		o		+/–	+	+/–	+	NHL
27			o		——		o	————		o		+/–	+	+		CLL
28	o		o	——			o	————		o		++	+			CLL
29			o		————					o		++	–	–		CLL
30	o			——			————					+/–	–			NHL
31	o			——			————					++	++			NHL
32				——	---	——	o	————				++				NHL
33	o			——			——	o	——			++	+++	+++		NHL
34	o		o	o				——	o	——		+	–		+	ALL
35	o		o					——		——		+	–			Plasmo.
36			o					——		——		+/–	–			PLL
37	o		o	——			————					–	–			CLL
38	o		o	————			o	---	——			–	+	–	+	CLL
39			o		——		o	o	——			+/–	–	+		CLL
40			o		o	——	---	o	——			–	+/–	+		CLL
41	o		o				---		——			–	+	–		PLL

—— = >20% of the cells positive;
--- = 10-20% of the cells positive;
o = <10% of the cells positive;
Blank = not done.

– = negative;
+/– = weakly positive;
+ = positive;
++ = highly positive;
+++ = very highly positive.

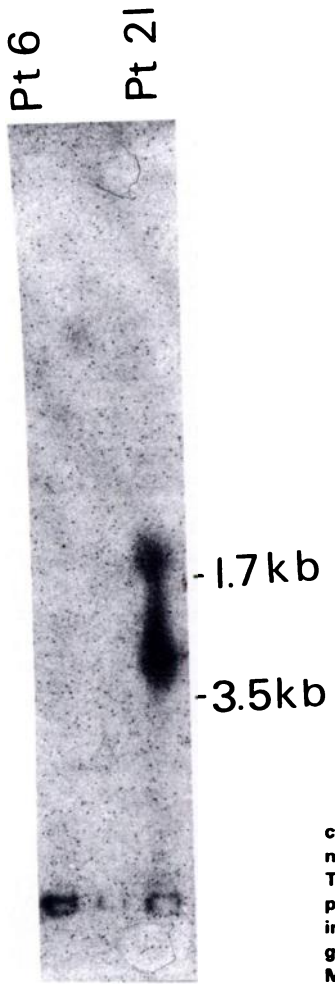

Fig 4. Northern blot of cytoplasmic RNA from patients no. 6 (lane 1) and 21 (lane 2) of Table 1 hybridized with a DNA probe for the J_H region of the immunoglobulin heavy chain gene (Oncor, Gaithersburg, MD).

leukemias carrying B-associated markers, μ mRNA is accumulated during early B-maturational steps. The highest levels of μ mRNA transcripts were present in leukemias at differentiation stages starting to express IgM on the cell surface.

It has been shown that μ mRNA may be transcribed not only from the completely rearranged immunoglobulin genes with the variable region gene segments, but even from truncated rearrangements,[22-24] as well as from unrearranged genes.[25-27] Such incomplete mRNAs can even be translated.[28,29] The significance of transcribed and translated incomplete μ chains with respect to further maturation along the B-cell axis is as yet unknown[16] and may be a characteristic of early B cells,[30] signalling the principal competence of the cells to synthesize the immunoglobulin heavy chain.

Our probes marking the constant regions of the mRNAs in situ do not say anything about the length of the mRNA. Therefore, in situ hybridization cannot provide information about whether the variable part of the receptor chains is included in the message or not.

Whereas in the acute leukemias progression of B-cell maturation was accompanied by a continuous increase in μ mRNA content, the group of chronic lymphatic diseases presented a different picture: Some of the CLLs carrying IgM on their surface were negative or only marginally positive for μ mRNA, others contained high amounts of μ mRNA, suggesting a second wave of differentiation. During the switch to other surface immunoglobulin heavy chains, μ mRNA content decreased. There were several CLLs that still expressed the μ chain on their surface but no longer transcribed the μ gene. Thus, μ chains may continue to be inserted into the cell membrane, even during very low transcriptional activity of the respective gene or when transcription has already ceased.

In all 12 B-determined leukemias tested for rearrangement of the μ gene, none was in germline configuration. Since only two thirds of these cases expressed μ mRNA, B-lineage determined leukemias that seem to be uniform with respect to rearrangement may further be subdivided by μ mRNA transcriptional activity.

TCR gene rearrangement has been reported in B CLLs[11,18,31] and rearrangement of both TCR and Ig genes has been observed simultaneously in some populations.[32,33] Therefore, we assayed some leukemic cell populations for TCRα and β mRNA expression and, in fact, TCR expression was found to occur in B-determined leukemias, as is evident from the fluorescence intensity histograms, even if only a limited number of populations were assayed. We detected unequivocal simultaneous transcription of TCR and μ genes as frequently (five of 22) as has been previously reported for simultaneous rearrangement of the two receptor genes (up to 27%).[4,11,17,20,22,32,33] There was no correlation between TCR mRNA expression and the negligible low levels of surface CD3 in any of the cases tested.

Especially in one case of NHL, high activation of all three genes investigated occurred simultaneously. The fact that all cells of this population were positive for all three receptor mRNAs revealed that simultaneous expression of the receptor genes for both the T and the B cell antigen receptors occurs in the same individual cells. These results are compatible with the observation that the same replication system is active in both T and B cells.[34] Whether such simultaneous activation also occurs under normal conditions or whether it is due to escape from normal control in the malignant cells, remains to be investigated. From our results, it is obvious that lineage cross-over transcription is not rare, and can occur even in leukemias ranked amongst the more mature ones, which has also been verified by Northern blots.

Thus, even if we have shown that receptor mRNA expression is not a more class-specific marker than either rearrangement or surface markers and cannot be used for definite classification of cell populations, it contributes additional information with respect to the biology and activity of the antigen receptor system in leukemias. If, in fact, the results obtained in malignant cells can be extrapolated to normal cells, this may open new aspects concerning antigen receptor expression in T and B lymphocytes. It would then be interesting for example to determine to what extent "wrong" receptor expression leads to cell elimination and clonal selection.

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Quantitative tracing of mRNAs for T- and B-lymphocyte receptor genes in individual cells by in situ hybridization with fluorochrome-labeled gene probes. I. Expression in malignancies carrying B-lineage associated antigens

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