**In Vitro and in Vivo Activation of B-Lymphocytes: A Flow Cytometric Study of Chromatin Structure Employing 7-Aminoactinomycin D**

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**ABSTRACT**

The chromatin structure of a diploid precursor B-cell line (REH), *in vitro*-stimulated normal B-lymphocytes, and reactive and malignant lymph node B-lymphocytes was studied by staining formaldehyde-fixed, permeabilized cells with the DNA-specific fluorophore 7-aminoactinomycin D (7-AMD) and measuring single-cell fluorescence by flow cytometry. Resting peripheral blood B- and T-lymphocytes (Go cells) bound low amounts of 7-AMD (7-AMD* phenotype), while G1 REH cells and purified B-cells stimulated with anti-μ+ B-cell growth factor bound nearly twice as much 7-AMD (7-AMD* phenotype). 7-AMD binding increased up to threefold and the differences in binding between Go and G1 cells were nearly abolished when nuclei were isolated prior to fixation or when fixed whole cells were treated with DNase 1. 7-AMD binding increased in parallel with autophosphorylation and approximately linearly with time during the Go→G1 transition of *in vitro* stimulated B-cells, as was determined by simultaneous measurements of 7-AMD fluorescence and autofluorescence or fluorescence of fluorescein isothiocyanate-labeled antibodies to the early activation antigen 4F2 and to the transferrin receptor.

In cell suspensions from lymph node biopsies, the 7-AMD* phenotype was a property of tumor cells in patients with high grade non-Hodgkin’s lymphoma (H-NHL, Kiel classification, 5/5); cells with this phenotype were only found in one of nine low grade non-Hodgkin’s lymphoma samples (L-NHL, 1/9). The other (8/9) L-NHL samples and the reactive samples (L-NHL, 1/9) had 7-AMD* cells. All tumors were diploid. The correlation observed between 7-AMD binding and DNase 1 susceptibility of DNA in chromatin (P < 0.001) suggests that 7-AMD binding is a marker of general transcriptional activity. Surprisingly, the percentage of tumor cells in S phase did not correlate significantly with 7-AMD stainability (P = 0.07), while the light scattering (cell size) of Go/G1 cells was highly correlated to 7-AMD binding (P < 0.001).

**INTRODUCTION**

During the last decade, a number of B-lymphocyte specific surface antigens have been defined (1). These antigens may also show activation/differentiation specific expression (2, 3). Expression of such antigens and/or clonal rearrangement of immunoglobulin genes in tumor cells have shown that most malignant lymphomas and chronic leukemias are of B-cell origin.

Patients with H-NHL have a very poor prognosis if untreated. Approximately 50% of the patients, however, can be cured with aggressive chemotherapy. The course of the disease and the response to chemotherapy is much more unpredictable in L-NHL which renders the choice of treatment for this tumor group difficult (4). We are accordingly searching for reliable markers of cell activation/proliferation which, besides histological grading, can give information on the biological aggressiveness of the lymphomas. Examples of biological parameters which have been linked to histological grading and/or to patient outcome are DNA synthesis ([3H]thymidine uptake, labeling index), cell cycle distributions, cellular volume and expression of activation antigens (5–14).

The chromatin of lymphocytes disperses on the ultrastructural (15) and microscopic (16) level upon stimulation. This change in appearance is preceded by changes on the molecular level. Darzynkiewicz et al. (17) demonstrated by acridine orange staining that the DNA in chromatin of stimulated lymphocytes (G1, S, G2) was less susceptible to acid-induced denaturation than that of resting lymphocytes (G0) and mitotic lymphocytes. Rabinovitch et al. (18) noted that interleukin 2 receptor* T-lymphocytes bound more 7-AMD than interleukin 2 receptor* cells in stimulated cultures.

Active chromatin, in addition to being more accessible to DNA binding chromophores, is also more sensitive to the action of nucleases like DNase 1 (19–21). Active genes reside in chromatin domains of the order of 100 kilobases which are generally more sensitive to DNase 1 than bulk chromatin (19).

In a previous study (22), we showed that peripheral blood myeloid cells bound more 7-AMD than lymphocytes. The binding of 7-AMD correlated with DNase 1 susceptibility, RNA synthesis and RNA content in these cells. Hence, it was suggested that 7-AMD binding was a measure of transcriptional activity in peripheral leucocytes. We report here on studies of the binding of 7-AMD in resting peripheral blood B- and T-cells, activated B-cells, a pre-B-cell line, as well as malignant and normal lymph node B-cells. The increased 7-AMD staining of the tumor cells in all patients with H-NHL resembled that seen with *in vitro* activated B-cells, indicating that these tumor cells exhibit an “activated” or “G1* like phenotype in vivo.

**MATERIALS AND METHODS**

Cells. The precursor B-cell line REH originating from a patient with acute lymphoblastic leukemia was kindly provided by M. F. Greaves, Imperial Cancer Research Fund Laboratories, London, UK. The cells were grown in RPMI 1640 (GIBCO, Paisley, UK) supplemented with 10% FCS at 37°C in 5% CO₂. PBL were prepared by centrifugating blood over Lymphoprep (Nyegaard, Oslo, Norway). Highly enriched resting human B-cells were isolated from peripheral blood buffy coats by a method developed in our laboratory (23). Briefly, Dynabeads (Dynal, Oslo, Norway) were coated with the CD19-specific antibody AB-1 (24) and added to buffy coats at a ratio of ten beads per B-cell. The mixture was incubated on a rocking platform for 30 min at 4°C. Cells forming rosettes with AB-1 beads were trapped by a samarium cobalt magnet. The non-rosetting cells were subsequently removed by suction. Rosetted cells were washed five times to remove nonrosetting cells. AB-1 beads were detached from the B-cells by overnight culture in RPMI 1640 supplemented with 1% FCS. This procedure yields less than 1% T-cells and monocytes as judged by indirect immunofluorescence staining with the pan-T antibody OKT 11 (Ortho Diagnostics Systems, Raritan, NJ) and the anti-monocyte antibody 1D5 (25). The B-cells were stimulated with 75 μg/ml F(ab')₂ fragments of rabbit IgG.
polyclonal antibodies to human μ heavy chain (DAKO, Copenhagen, Denmark), prepared by pepsin treatment as described (26) and with partially purified human low molecular weight B-cell growth factor (BCGF, Cellular Products, Buffalo, NY) at a 1:5 dilution in RPMI. Cell suspensions from lymph node biopsies of patients with histologically (Kiel classification) and immunologically (27) verified non-Hodgkin's lymphomas were prepared as described elsewhere (6). The tumor populations of these suspensions were all shown to be of B-cell origin as mononuclear light chain restriction for one of the two light chain isotypes was observed (more than 95% predominance). The cell suspensions contained both malignant and reactive cells, but the malignant B-cell fraction always exceeded 60% of the total cell number (see Table 1). Also included is a cell suspension of a reactive lymph node.

Antibody Labeling. Cells were labeled with FITC-conjugated rabbit anti-human polyclonal antibodies to κ or λ light chain surface immunoglobulin (DAKO, Copenhagen, Denmark). The antigens recognized by monoclonal antibodies 4F2 (courtesy of Dr. S. Fauci) and B3/25 (transferrin receptor, courtesy of Dr. I. R. Trowbridge) was labeled by monoclonal antibodies 4F2 (courtesy of Dr. S. Fauci) and B3/25 (transferrin receptor, courtesy of Dr. I. R. Trowbridge) was labeled by addition of 100% methanol to the pellet, respectively. Cells and nuclei were fixed for at least 1 week at 0°C.

Fixation/Staining. Cells were washed once in phosphate-buffered saline, resuspended in phosphate-buffered saline and fixed by the addition of freshly prepared 4% phosphate-buffered formaldehyde to a final concentration of 3%. Nuclei were isolated by gently resuspending the pellet in 0.15 M NaCl, 0.5 mM EDTA, 10 mM phosphatase buffer (pH = 7.4), 0.1% Nonidet P40. 5 min later nuclei were vortexed and fixed as above (28). Cells were fixed in 70% ethanol and 100% methanol by addition of nine parts of 77% ethanol to cells in 0.15 M NaCl and by addition of 100% methanol to the pellet, respectively. Cells and nuclei were fixed for at least 1 week at 0°C.

Table 1 Histological grading, 7-AMD binding in G0/G1, phase, and percentages in S phase of cells employed in this study

<table>
<thead>
<tr>
<th>Case</th>
<th>Histologya</th>
<th>%B-cells</th>
<th>Normal cells</th>
<th>Tumor cells</th>
<th>%S</th>
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<tbody>
<tr>
<td>H-NHL group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150/87</td>
<td>LB</td>
<td>&gt;90</td>
<td>0.84</td>
<td>1.25</td>
<td>30</td>
</tr>
<tr>
<td>68/86</td>
<td>LB</td>
<td>90</td>
<td>0.85 ± 0.04(2)</td>
<td>1.58 ± 0.14(2)</td>
<td>21</td>
</tr>
<tr>
<td>151/82</td>
<td>CB</td>
<td>78</td>
<td>0.94</td>
<td>1.44</td>
<td>13</td>
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<tr>
<td>128/86</td>
<td>CB</td>
<td>64</td>
<td>0.85 ± 0.04(2)</td>
<td>1.56 ± 0.00(2)</td>
<td>12</td>
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<tr>
<td>398/85</td>
<td>IB</td>
<td>77</td>
<td>1.06</td>
<td>1.88</td>
<td>8</td>
</tr>
<tr>
<td>L-NHL group</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>164/78</td>
<td>CC/CB</td>
<td>87</td>
<td>=0.94</td>
<td>=1.38</td>
<td>5</td>
</tr>
<tr>
<td>337/85</td>
<td>CC/CB</td>
<td>74</td>
<td>1.00</td>
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<tr>
<td>73/87</td>
<td>CC</td>
<td>93</td>
<td>0.84</td>
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<tr>
<td>382/85</td>
<td>CC</td>
<td>81</td>
<td>0.93 ± 0.02(2)</td>
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<tr>
<td>339/79</td>
<td>CC</td>
<td>70</td>
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<tr>
<td>325/80</td>
<td>IC</td>
<td>82</td>
<td>0.88</td>
<td>3</td>
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<tr>
<td>60/84</td>
<td>LY</td>
<td>63</td>
<td>0.86</td>
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<tr>
<td>136/80</td>
<td>LY</td>
<td>80</td>
<td>0.81</td>
<td>1</td>
<td></td>
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<tr>
<td>62/87</td>
<td>CLL</td>
<td>95</td>
<td>1.09</td>
<td>5</td>
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</tr>
<tr>
<td>328/86</td>
<td>Reactive</td>
<td>45</td>
<td>0.88</td>
<td>3</td>
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<td>Other cells</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>PBL</td>
<td></td>
<td></td>
<td>0.89 ± 0.17(13)</td>
<td>&lt;1</td>
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</tr>
<tr>
<td>REH</td>
<td></td>
<td></td>
<td>1.53 ± 0.15(2)</td>
<td>35</td>
<td></td>
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<tr>
<td>Monocytes</td>
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<td>1.62 ± 0.06(2)</td>
<td>&lt;1</td>
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<tr>
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<td>0.88 ± 0.07(2)</td>
<td>&lt;1</td>
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<td>1.66 ± 0.10(2)</td>
<td>17</td>
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</table>

* Kiel classification.
# Relative to rat thymocyte nuclei.
^ Number of experiments.
instrument. RTN and 1.33-μm fluorescent microspheres (Polysciences, Warrington, PA) were also added to all samples as internal controls for H33258/7-AMD staining and light scattering, respectively. All H33258 and 7-AMD fluorescence values are given in units of fluorescence of RTN. The arrows in the figures point to the fluorescence of RTN (RTN and "dead" cells scatter less light than intact whole cells. Two-parameter histograms were gated on the light scattering of intact cells to exclude the peaks of RTN and "dead cells" in Figs. 1A, 1B, 2, 4, and 5). Light scattering is given in units of the light scattering of 1.33-μm fluorescent microspheres, which also served as internal control for FITC fluorescence. Immunofluorescence is given in units of fluorescence of 1.33-μm microspheres.

Data Treatment. 7-AMD fluorescence values given are peak values of G0/G1 peaks (relative to the peak position of RTN). The standard errors of the peak values of individual results were negligible. Results of multiple separate experiments are given as means of peak values (Table 1, Figs. 7 and 8). Deviations (Table 1) and bars (Figs. 7 and 8) represent the full range spanned by the data. In Fig. 3, median green fluorescence was calculated for each red fluorescence channel (64 × 64 channels). Autofluorescence was subtracted from 4F2 and B3/25 data. Data were normalized for late S/G2 + M cells. The scaling factors are given in Fig. 3, legend. The high number of data points allowed the drawing of smooth curves. The G0/G1 border indicated in Fig. 3 was determined from the histogram obtained by projecting the data onto the 7-AMD axis (similar in appearance to that shown in Fig. 1A, note the position of RTN). The border was set in the valley between the G0 and G1 peaks such that more than 95% of unstimulated cells were defined as "G0" cells. The percentages of cells in G0 + G1, S, and G2 + M were determined from an H33258 fluorescence histogram of the same sample of cells (before addition of 7-AMD, similar in appearance to that shown in Fig. 1B). These percentages were used to set the G0 + G1/S and S/G2 + M borders in Fig. 3. This procedure rests on the assumptions that S-phase cells have more 7-AMD fluorescence than G1 cells and that G0/M cells have more 7-AMD fluorescence than S-phase cells.

Estimation of P values (two-tailed probabilities for the correlation between two parameters being purely accidental, Figs. 6–8) was performed according to Student's t test. P values less than 0.05 were assumed to indicate statistical significance. The solid lines in Figs. 6–8 are the least-square linear regression lines.

The border between the "7-ADM++" and the "7-AMD--" phenotypes (7-AMD fluorescence = 1.17, horizontal lines in Figs. 7 and 8) was set such that PBLs and resting B-cells were negative. Additionally, it was required that in all cases where two peaks were evident in histograms (Figs. 1A, 4B, and 4C, and cases 128/86, 151/82, and 398/85), the cells with lower and higher fluorescence should be negative and positive, respectively.

RESULTS

As an in vitro model for activated B-lymphocytes we employed anti-μ + BCGF-stimulated purified B-cells and the pre-B-cell line REH. Fig. 1A shows a fluorescence histogram of a mixture of unstimulated B-cells and B-cells stimulated with anti-μ + BCGF for 72 h. The binding of 7-AMD increased approximately twofold during the G0-G1 transition of B-cells (Table 1). The same difference in 7-AMD binding was observed between PBL and G1 REH cells (Table 1). Pretreatment with RNase had no effect on 7-AMD stainability (data not shown), 7-AMD binding was up to 200% higher in fixed nuclei compared to fixed whole cells (compare Fig. 1C with 1A, data not shown for REH and PBL). The difference in 7-AMD binding between G0 and G1 nuclei was approximately 25% (Fig. 1C, data not shown for REH and PBL), i.e., much smaller than the difference between G0 and G1 whole cells. The effects of fixation with 70% ethanol on 7-AMD stainability were investigated with REH cells and PBL. G1 REH cells bound 6.25 RTN units of 7-AMD, while PBLs bound 4.75 RTN units of 7-AMD. Hence, binding was much higher than in formaldehyde-fixed cells (see Table 1), but the difference in binding between G1 REH cells and PBL was smaller (=30%). 100% methanol-fixed cells bound the same amount of 7-AMD as 70% ethanol-fixed cells.

Staining of chromatin with H33258 was stoichiometric under these conditions (Fig. 1, B and D, data not shown for REH, PBL, and lymphomas). Ploidies and percentages of cells in the G0 + G1, S and G2 + M phases of the cell cycle could therefore be estimated from H33258 histograms. It was also noted that H33258 staining was independent of fixation procedure; the stainability varied by less than 10% between cells fixed with paraformaldehyde (cells and nuclei), 70% ethanol, 100% methanol and unfixed nuclei. The coefficients of variation of the G0 + G1 peaks, however, were noticeably lower for the H33258 distributions of formaldehyde-fixed nuclei [Fig. 1D, mean (± SD) = 2.3% (±0.9%) for nine representative histograms of REH, PBL, B-cells, stimulated B-cells and lymphomas] than for the corresponding of whole formaldehyde-fixed cells [Fig. 1B, mean (± SD) = 3.7% (±1.8%)]. These coefficients of variation were mostly due to heterogeneity in cellular fluorescence intensity, as instrumental resolution was below 1%.

A nearly twofold higher binding of 7-AMD in monocytes relative to lymphocytes was associated with increased transcriptional activity as measured by RNA synthesis, RNA content, and DNase I susceptibility (22). Fig. 2B shows that the chromatin of REH cells was more susceptible to DNase I degradation than that of PBLs as measured by H33258 fluorescence. Interestingly, and in agreement with earlier results on leucocytes, 7-AMD binding increased after DNase I treatment (Fig. 2A). 7-AMD binding increased much more in PBL than in REH, resulting in nearly equal fluorescence from G0 (PBL) and G1 (REH) cells. Higher transcriptional activity is therefore probably the reason for the higher binding of 7-AMD in G1 B-cells and REH cells relative to resting B-cells and PBL (RNA synthesis increases 7-fold during the G0-G1 transition of B-cells). The kinetics of the increase in 7-AMD binding during the transition of B-cells through the cell cycle was indirectly studied by measuring 7-AMD fluorescence versus fluorescence from...
FITC-labeled antibodies to activation antigens or autofluorescence. Autofluorescence was assumed to be proportional to total cellular protein content. A heterogeneous sample of anti-μ + BCGF stimulated B-cells containing G0, G1, S, G2, as well as cells in the G0/G1 transition was employed for this study. 7-AMD binding increased in parallel with autofluorescence during the transition from G0 to late G1 (Fig. 3, solid line, top).

The 4F2 antigen is expressed on the surface of B-cells approximately 4 h after stimulation, while the transferrin receptor is expressed after 20 h (14). These estimates indicate that early G1 (as measured by 7-AMD fluorescence, left part of "G1" column) is reached 4 h after stimulation, while cells pass through "middle" G1 after approximately 20 h. [3H]Thymidine is incorporated after 35–40 h (31). Hence, 7-AMD binding increased approximately linearly with time after stimulation.

Single cell suspensions from lymph node biopsies were fixed and stained with 7-AMD to see whether malignant B-lymphocytes showed distinctive G0/G1 binding patterns. The sample material included lymph nodes of 14 lymphoma patients and one benign hyperplastic lymph node (see Table 1). All lymphomas were of B-cell origin. Most L-NHL lymphoma cell suspensions (8/9) and the normal lymph node sample contained only cells which bound "normal" amounts of 7-AMD in the G0/G1 phase ("7-AMD": fluorescence <1.17, Table 1, Figs. 7 and 8). In such cases a unimodal peak with a coefficient of variation from 8 to 13% was evident. The large coefficients of variation were due to staining heterogeneity and not to lack of instrumental resolution. A typical example of this group is shown in Fig. 4. "Dead" cells bound more 7-AMD and scattered less light than cells with intact cell membranes at the time of fixation (data not shown). "Dead" cells were excluded on this basis, and the results refer to properties of "live" cells. Simultaneous labeling of the tumor characteristic light chain was performed to show that the tumor cells in case 382/85 were 7-AMD+ (as described in "Materials and Methods," data not shown).

Samples from five cases of H-NHL contained a subpopulation of cells which bound more 7-AMD in the G0/G1 phase ("7-AMD": fluorescence >1.17, Table 1, Figs. 7 and 8). Two separate peaks were observed in these 7-AMD histograms; in all five cases the fluorescence of 7-AMD+ cells resembled that observed with PBL or unstimulated B-cells (Table 1). The 7-AMD+ cells scattered light as PBL; these cells are probably normal lymphocytes present in these samples. Fig. 4, B and C, shows representative examples of 7-AMD fluorescence patterns obtained for this group. The tumor cells were 7-AMD+ in cases...
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Fig. 4. Cells from biopsies 73/87 (A), 68/86 (B), 150/87 (C), and 164/78 (D) were fixed and stained with H33258. The H33258 fluorescence distributions were measured (histograms not shown), 7-AMD was added and measurements were repeated with 578-nm excitation to give 7-AMD fluorescence distributions (gated on light scattering).

Fig. 5. Whole cells from biopsy 164/78 were labeled with antibodies to κ or λ light chains, fixed, and stained with 7-AMD. 7-AMD versus FITC fluorescence distributions were measured with 488-nm excitation. 7-AMD fluorescence distributions are shown for κ+ and κ− cells. Tumor cells have κ+ phenotype. Only a few κ−/7-AMD+ cells could be identified in the anti-κ stained sample which served as control (not shown).

Fig. 6. The 7-AMD fluorescence of fixed G0/G1 cells (x, △, □, ▢) or nuclei (○, △) was measured and plotted against the DNase 1 sensitivity of PBL (x, △), REH (○, △), stimulated B-cells (□) and normal and neoplastic cells in samples from biopsies 128/86, 398/85, 150/87, and 151/82 (○). "DNase 1 sensitivity" was defined as 1−[I(H33258)DN]/I(H33258)0], where I(H33258)0 was the G0/G1 peak H33258 fluorescence of untreated cells, while I(H33258)DN was the H33258 fluorescence of cells digested for 30 min with DNase 1 as described in "Materials and Methods."

The tumor cells were diploid within 10% in all cases (data not shown). Differences in 7-AMD binding can therefore not be explained by differences in ploidy.

128/86 and 68/86 (the tumor cells were identified by simultaneous labeling for predominant light chains as described in "Materials and Methods," data not shown). The number of cells binding high amounts of 7-AMD in cases 150/87, 151/82, and 398/85 corresponded well with the high number of B-cells in these samples (Table 1), suggesting that the 7-AMD+ phenotype is a property of tumor cells also in these cases.

One case (164/78, Fig. 4D), representing a centroblastic/centrocytic lymphoma, showed a bimodal 7-AMD fluorescence distribution. Simultaneous measurement of light chain expression and 7-AMD fluorescence was performed; the 7-AMD fluorescence distributions of κ+ and κ− cells given in Fig. 5 show that 7-AMD+ cells were of tumor cell origin. Note the lack of a 7-AMD+ peak in the histogram of κ− cells and the presence of 7-AMD+/κ− cells, presumably representing the centrocytic compartment of the tumor and/or normal lymph node B-cells.

The tumor cells were diploid within 10% in all cases (data not shown). Differences in 7-AMD binding can therefore not be explained by differences in ploidy.

It was investigated whether the 7-AMD stainability correlated with other parameters. Correlation with DNase 1 susceptibility was good (Fig. 6). Surprisingly, no significant correlation was found between 7-AMD stainability and the percentage of tumor cells in S phase (Fig. 7). 7-AMD binding was, however, correlated with the light scattering of G0/G1 cells (Fig. 8). Data for case 164/78 were not used in these estimates, due to the large uncertainties in the determination of peak values (Fig. 4D).

DISCUSSION

By using in vitro and in vivo B-cell systems, we have shown that increased binding of the DNA-specific dye 7-AMD is a reliable characteristic of "activated" B-lymphocytes. In a previous communication (22), we presented evidence that 7-AMD binding was a measure of transcriptional activity in peripheral leucocytes. The higher transcriptional rate of in vitro-stimulated B-cells and REH cells compared to resting lymphocytes and the high correlation between DNase 1 susceptibility and 7-AMD binding (Figs. 2 and 6) indicates correlation between transcriptional activity and 7-AMD binding also in the in vitro and in vivo B-cell systems employed in this study. Hence, "activation" is assumed to be synonymous to increased general transcriptional activity in this context.

The molecular mechanisms responsible for the phenotype-specific restriction of 7-AMD to DNA in formaldehyde-fixed intact chromatin are not known at present. Presumably, some nuclear factor(s) create(s) the differential chromatin structures of resting and activated lymphocytes. The differential effects exerted on 7-AMD stainability of these factors were markedly

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Fig. 7. The 7-AMD fluorescence of fixed G0/G1 whole cells was measured and plotted against percentage of S-phase cells as determined from the H33258 fluorescence distribution of whole cells or nuclei. Results are shown for PBL/resting B-cells (A), stimulated B-cells (T), monocytes (•), REH cells (●), and neoplastic cells of all lymphomas (except for 164/78) listed in Table 1 (x).

Reduced by extracting nuclei with detergent before fixation (Fig. 1C), by fixing cells with 70% ethanol or 100% methanol or by post fixation with DNase 1 (Fig. 2). The possible functions of such factors were discussed in more detail in a previous work (22).

The fluorophore 7-AMD, in addition to being a sensitive probe for chromatin structure, has attractive optical properties: Fluorescence of the DNA-bound dye is in the deep red, resulting in no overlap with FITC fluorescence. Since 7-AMD fluorescence may be excited at 488 nm, antigen expression may be measured simultaneously (Figs. 3 and 5). In fact, the fluorescence of two different antibodies (conjugated to FITC and phycoerythrin, respectively) has been measured simultaneously with 7-AMD fluorescence (18). Double labeling with FITC-conjugated antibody and 7-AMD was performed in this work to: (a) Identify the tumor cell populations in biopsy samples (note that although monotypic light chain expression is a characteristic of B-cell tumors, such expression is not specific for tumor cells. The fractions of neoplastic B-cells, however, were so high for the biopsy samples employed here that identification was straightforward.), (b) Determine the relationship between the increased binding of 7-AMD and the acquisition of surface activation/differentiation antigens during \textit{in vitro} B-lymphocyte traverse of the cell cycle (Fig. 3). The kinetics of the appearances of the antigens 4F2 (early G1) and transferrin receptor (late G1) are known (14). The presented method may be used to determine how other uncharacterized antigens are acquired or lost during \textit{in vitro} or \textit{in vivo} activation of cells.

The total protein content (autofluorescence) increased approximately linearly with 7-AMD fluorescence during the G0—G1 transition of stimulated B-cells (Fig. 3). Assuming that 7-AMD is largely proportional to the amount of active chromatin, this indicates that the number of active genes increases steadily during activation of B-cells from the quiescent state.

Staining with 7-AMD proved to be useful for detection of \textit{in vivo} activation of malignant B-lymphocytes. Interestingly, all lymph nodes (5/5) from patients with H-NHL contained a subpopulation of 7-AMD* cells (Table 1, Fig. 4, A and B). In contrast, nearly all samples from L-NHL patients (8/9) and the benign hyperplastic lymph node contained only 7-AMD* cells. One L-NHL case, 164/78, showed a bimodal 7-AMD fluorescence distribution (Fig. 4D); most tumor cells were 7-AMD* also in this case (Fig. 5). As this case represents a centroblastic/centrocytic lymphoma, the 7-AMD* cells probably represent the centroblasts. It appears that the 7-AMD* phenotype is characteristic of cells morphologically resembling "blasts," typically present in lymph nodes/peripheral blood of patients with high grade malignant lymphoid disorders. The large size of these cells also supports this notion (Fig. 8).

On the other hand, 7-AMD does not discriminate between indolent and more aggressive L-NHL as do other parameters of cell activation like [3H]thymidine uptake and expression of the 4F2 antigen (14). Determination of the 7-AMD phenotype may, however, be useful for detecting the transformation of L-NHL to H-NHL in repeat biopsies during, e.g., prolonged observation of untreated patients.

The correlation between 7-AMD binding and DNase 1 susceptibility indicated that increased transcriptional activity was the cause of increased 7-AMD binding also in \textit{in vivo}-activated B-cells (Fig. 6). The better correlation between 7-AMD binding and light scattering (Fig. 8) than that between 7-AMD binding and percentage of S-phase cells (Fig. 7) have the following general implications: (a) Large cells are transcriptionally more active than small cells (not unexpected). (b) Cells may enter S
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phase without showing any signs of “activation” as indicated by the acquisition of the 7-AMD* phenotype (382/85, 73/87, 328/86). (c) Cells may be “activated” without subsequent entry into S phase (monocytes, note also that the G0/G1 tumor cells in case 398/85 bound most 7-AMD, while only 8% of these cells were in S phase). There are many examples of cells behaving according to implication (c). Carlsson et al. (32) studied a B-chronic lymphocyte leukemia clone which differentiated without proliferation into IgM-secreting plasma blasts/lymphoblasts after 12-O-tetradecanoylphorbol-13-acetate stimulation [T-cell-derived factors could provide the additional signals required for proliferation to occur]. Anti-IgM in submictogenic concentrations and the anti-CD20 antibody 1F5 trigger B-cells to enter G1, but have very low mitogenic effects (33).

We propose that the methods reported here can be useful for diagnostic purposes as indicated as well as for simultaneous measurements of cell cycle traverse and antigen expression in lymphoid systems. It remains to be seen whether 7-AMD binding can be used as a marker of transcriptional activity in other cell types.

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In Vitro and in Vivo Activation of B-Lymphocytes: A Flow Cytometric Study of Chromatin Structure Employing 7-Aminoactinomycin D

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