Heterogeneity of Non-Hodgkin's Lymphoma Probed by Nucleic Acid Cytometry

By John Srigley, Bart Barlogie, James J. Butler, Barbara Osborne, Mark Blick, Dennis Johnston, Hagop Kantarjian, James Reuben, John Batsakis, and Emil J Freireich

Flow cytometric analyses of cellular DNA, RNA, and double-stranded RNA content were performed on lymph nodes and extranodal tissue from 177 patients with non-Hodgkin's lymphoma. With increasing histologic grade, a higher incidence of aneuploidy, higher proliferative activity, and higher total and double-stranded RNA content were found. Despite considerable cytometric heterogeneity within histologic grades and morphologic subtypes, conformity between cytometric and morphologic classifications was observed in 85% of cases. Among intermediate-grade and high-grade lymphomas, increased proliferative activity and diploidy were associated with more frequent responses to treatment. Thus, nucleic acid-derived parameters relate to morphologic subtypes and permit an objective approach to lymphoma classification based on ploidy, proliferation, and RNA characteristics that also had prognostic implications.

THE NON-HODGKIN'S lymphomas (NHL) represent a spectrum of hematologic malignancies characterized by a highly diverse clinical behavior. Although morphologic approaches have been subject to technical and interpretative problems, prognostically relevant classifications based on growth pattern and cell size have evolved.1,6 The international working formulation provides a framework for comparison among these most commonly used systems.7 Advances in immunology have made possible the phenotypic characterization of NHL in relationship to normal lymphocyte differentiation and maturation.8 At least two of the current taxonomic schemes emphasize a combined immunologic-morphologic approach,9,4 but the independent prognostic relevance of phenotypic characteristics has not been established.9

The biologic and clinical heterogeneity of NHL is contributed to by factors other than structural and cell surface characteristics. Included among these are genetic, kinetic, and metabolic features. Several investigative approaches including classical cytogenetics,10-15 tritiated thymidine labeling,16-18 and DNA flow cytometry (FCM)19-29 have been used to explore these biologic variables. With use of appropriate fluorescent dyes, nucleic acid FCM provides objective and quantitative measurements of macromolecules in a rapid and statistically applicable manner. Smaller studies of NHL using DNA cytometry have shown that aggressive lymphomas are associated with more frequent ploidy abnormalities and higher proliferative activity than are indolent lesions.20,21,25-28,29

We have evaluated FCM as a quantitative cytologic tool to measure ploidy and cell cycle distribution, as well as total and double-stranded RNA content, in 177 patients with NHL. Our results indicate a high degree of conformity between certain cytometric and morphologic features. In addition, ploidy and cytokinetic findings correlated with the initial response to treatment and survival.

MATERIALS AND METHODS

Study Population

One hundred eighty-five samples from 177 patients with NHL, including 76 at diagnosis and 109 at relapse (23 with histologic transformation), were studied (Table 1). Samples were lymph nodes in 157 cases and extranodal tissue in 28. Representative tissue was obtained at biopsy using frozen-section guidance. In 17 instances, samples were obtained by fine-needle aspiration, using multiple passes. In these instances, histologic material taken within 48 hours of the aspiration was also available. As controls, 27 nonepithelial lymph node samples were used. The lymphomas were classified according to the International Working Formulation scheme7 and grouped into low-grade (76 cases), intermediate-grade (90 cases), and high-grade histologies (19 cases).

Lymphocyte Marker Studies

Immunologic classification was available in 104 patients, using monoclonal antibodies against T and B cell antigens as well as heavy- and light-chain antisera for surface immunoglobulin analysis as previously described.30,31 Immunologic phenotyping was conducted by measuring the fluorescence intensity of the aforementioned reagents with an Ortho Spectrum III flow cytometer (Ortho Instruments, Westwood, Mass). B cell lymphoma was diagnosed when >70% of cells expressed a single heavy-chain class or B-1 positivity or when monotypic light-chain expression was noted (λ/κ > 3 and λ/κ > 2). Similarly, T cell disease was confirmed when >70% of cells expressed at least one monoclonal T cell marker. The exception here was the presence of extensive Leu-1 positivity in small lymphocytic lymphomas of B cell type. In ten instances, frozen section immunoperoxidase studies were performed, using a similar panel of antibodies.32,33 The remaining patients were placed in an indeterminate category.
samples were placed in RPMI 1640 growth medium (GIBCO, Grand Island, NY) and immediately refrigerated. The sample preparation and analysis were completed within four hours. Biopsies were minced mechanically with surgical blades and sieves and syringed through progressively smaller needles until a single-cell suspension was available. For needle aspirates, syringing was the sole mechanical procedure. Specimens were then washed in phosphate-buffered saline (PBS) and adjusted to a concentration of 10^6 cells per cubic centimeter using a Coulter counter (Coulter Electronics, Hialeah, Fla). Two to 10 x 10^6 cells were obtained from each sample. When sufficient cells were available, an aliquot was fixed in 70% ethanol and refrigerated at 4 °C for high-resolution DNA and double-stranded RNA analysis.

For simultaneous DNA–RNA analysis, cells were stained with acridine orange according to a two-step procedure. FCM analysis was carried out with an ICP-22 mercury-arc based cytometer (Phywe, Goettingen, Germany) using appropriate excitation and emission filters. At least 10,000 cells were measured in 90% of samples; the minimum number was 4,000 cells. For DNA-derived ploidy analysis, samples were mixed with normal diploid peripheral blood lymphocytes obtained after Ficoll-Hypaque gradient separation, and the DNA index (DI) was computed, using the ratio of relative DNA contents of test and reference G,0 cells. For the analysis of the proliferative compartment, a “boxogram” gating procedure along the G1,S interface was used, and the percentage of cells in the (S + G2,M) cell cycle compartments was obtained. For DNA aneuploid populations, the (S + G2,M) compartment could be computed in 40% of cases because the populations were separable on the basis of their RNA content. In the remaining cases, aneuploid and diploid were not separable on the basis of RNA features; in these instances, an overall proliferative activity of the mixed population was determined. An RNA index was derived of the G1,S compartment according to criteria previously established, i.e., the ratio of the mean RNA content of the sample G,0 population and the median RNA content of lymphocyte controls. In aneuploid cases, the RNA index of the DNA-abnormal population was used.

When enough cells were available, aneuploidy as determined by the acridine orange technique was confirmed by high-resolution DNA analysis, using the ethidium-bromide/mithramycin dye combination and an ICP-11 mercury-arc–based cytophotometer (Phywe). In 40 patients with lymphoma and in 11 controls, double-stranded RNA (ds-RNA) was measured by using a technique originally developed by Frankfurt. Ethanol-fixed cells were washed in PBS, resuspended in DNase, and incubated at 37 °C for 40 minutes. After DNA digestion, cells were stained with propidium iodide and measured with an EPICS V cell sorter (Coulter). Ficoll-Hypaque- and RNA features of patients studied at diagnosis and relapse without histologic transformation were similar when analyzed grade for grade. Patients with histologic transformation had a higher incidence of aneuploidy than de novo intermediate- and high-grade lymphomas (P = .04), but showed similar proliferative and RNA features. There was no relationship between stage of disease and cytometric parameters when they were analyzed grade for grade.

Collections of codified data were compared using parametric (t test, analysis of variance, correlation) and non-parametric procedures (Mann-Whitney, Kruskal-Wallis, chi-square). A discriminant function was used to determine the concordance between morphologic and cytometric data. Survival of subgroups was compared using a proportional hazards method.

RESULTS

General Cytometric Features

Aneuploidy (DI 1) was observed in 37% of 185 cases. DNA abnormalities clustered in the low-degree hyperdiploid and tetraploid regions. There were only four hypodiploid DNA stemlines and three instances of multiple DNA-abnormal clones. Ploidy, proliferative, and RNA features of patients studied at diagnosis and relapse without histologic transformation were similar when analyzed grade for grade. Patients with histologic transformation had a higher incidence of aneuploidy than de novo intermediate- and high-grade lymphomas (P = .04), but showed similar proliferative and RNA features. There was no relationship between stage of disease and cytometric parameters when they were analyzed grade for grade.

Samples from extranodal sites had higher (S + G2,M)% values than those from nodal sites, 17.7% compared to 9.4% (P < .01), but ploidy and RNA features were similar. This proliferative difference reflects the high proportion of intermediate and high-grade lymphomas in extranodal sites. There were no significant cytometric differences between samples obtained by tissue biopsy and fine-needle aspiration.

Cytometry and Histologic Grade

Low-grade lymphomas showed a low frequency of aneuploidy, 17% compared with 52% and 47% for the intermediate- and high-grade lesions, respectively (Table 2). Even though overlap was present, the mean proliferative activity increased progressively from 4.7% for low-grade lymphomas to 12.5% for intermediate-grade, and to 26.2% for high-grade lymphomas (Fig 1). RNA index values differed between low-grade, intermediate-grade, and high-grade lymphomas. The “RNA-proliferation maps” demonstrated differences in the location, dispersion, and axis of the confidence ellipses among histologic grades (Fig 2).

<table>
<thead>
<tr>
<th>Histologic Grade</th>
<th>Status at Biopsy</th>
<th>Stage</th>
<th>Biopsy Site</th>
<th>Biopsy Mode</th>
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<tbody>
<tr>
<td>Low</td>
<td>Diagnosis</td>
<td>Relapse</td>
<td>Transformed</td>
<td>I, II</td>
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<tr>
<td></td>
<td>35</td>
<td>41</td>
<td>0</td>
<td>11</td>
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<tr>
<td>Intermediate</td>
<td>35</td>
<td>33</td>
<td>22</td>
<td>28</td>
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<tr>
<td>High</td>
<td>6</td>
<td>12</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
<td>86</td>
<td>23</td>
<td>42</td>
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</table>
Mean ds-RE correlated well with histologic grade: there was a progressive increment with transition from low- to intermediate- to high-grade lymphomas (Table 2). Although normal lymph node samples were diploid and manifested proliferation and RNA index features that were indistinguishable from low-grade lymphoma, the mean ds-RE values were significantly higher in low-grade lymphoma as compared with benign controls ($P < .01$). In lymphomas in which aneuploid and diploid cell populations were separable by RNA features, the mean $(S + G_2M)\%$ values of the diploid subpopulation was significantly higher than that of nonneoplastic controls (9.6% v 5.6%; $P < .01$).

**Cytometric and Specific Histologic Subtypes**

Cytometric features of individual histologic subtypes of NHL are detailed in Table 3. Small lymphocytic lymphoma showed a 7% frequency of aneuploidy and low mean proliferative and RNA features. The frequency of DNA-abnormal stemlines increased with the proportion of large cells in the follicular lymphomas with rates of 21%, 37%, and 64% in small cleaved-, mixed-, and large-cell varieties, respectively. In addition, increments in $(S + G_2M)\%$ and RNA index were noted as the proportion of large cells increased. Follicular and diffuse large-cell lymphomas shared similar aneuploidy rates, but the diffuse lesions had significantly higher $(S + G_2M)\%$ values (15.2% v 8.0%; $P < .01$). There were also higher values of $(S + G_2M)\%$ and RNA index in diffuse large- v diffuse mixed-cell lymphomas (only seven cases), but this difference was not significant. A comparison between follicular and diffuse, small, cleaved-cell lymphomas revealed similar aneuploidy rates (21% and 17%, respectively), but markedly higher proliferative activity in the latter type (4.4% v 8.8%; $P < .05$). In the high-grade category, all four immunoblastic sarcomas were aneuploid and showed higher mean $(S + G_2M)\%$ and RNA index values than nonimmunoblastic diffuse, large-cell lymphomas. The immunoblastic sarcomas had the highest mean RNA values of any histologic subtype. Lymphoblastic and small, noncleaved (undifferentiated) lymphomas showed aneuploidy rates of 50% and 37%, and similar proliferation and RNA features. The four AIDS-related lymphomas (three small noncleaved and one diffuse large-cell) were all diploid and had very high proliferative and RNA features.

**Cytometric Classification of Malignant Lymphoma**

By discriminant analysis, employing three acridine orange-derived parameters (aneuploidy, $(S + G_2M)\%$, RNA index), the morphologic grade was correctly predicted in 67% of 152 cases (Table 4). When the same analysis was undertaken in 40 patients with additional information on ds-RE values, the prediction of grade was improved to 85%. The ds-RE parameter alone allowed discrimination among grades in 59% of cases. No low-grade lymphoma was predicted as a high-grade lymphoma or vice versa.

**Cytometry and Immunophenotype**

Aneuploidy rates for the 71 B cell and six T cell lymphomas were 30% and 33%, respectively. Lymphomas of indeterminate phenotype had a higher frequency of abnormal DNA stemlines (46% of 27 cases) which was accounted for, at least in part, by a higher proportion (78%) of intermediate- and high-grade lymphomas.
histologic classes. Proliferation and RNA parameters showed no correlation with surface marker status.

**Prognostic Implications of Cytometric Findings**

Among the intermediate- and high-grade lymphomas, 33 patients studied at diagnosis and 57 at relapse were evaluable for response to chemotherapy (Table 5). High proliferative activity at diagnosis (S + G2M% > 15) was associated with a higher complete remission (CR) rate (77% vs 41%; P = .06). The presence of aneuploidy was an unfavorable feature for attainment of CR in previously treated patients (9% vs 32%; P = .03). Multivariate regression analysis for survival identified only prior treatment status and high proliferative activity as independent factors adversely affecting survival time.

**DISCUSSION**

We have demonstrated in a group of 177 patients with NHL that conventional morphologic subgroups have distinctly different ploidy and proliferative characteristics as assessed by DNA flow cytometry. This observation is in accord with previous smaller studies using other DNA fluorochromes.20-21,25-29 The strong correlation between proliferative activity and histologic grade in NHL is also in keeping with S-phase labeling studies using tritiated thymidine.16,17 The total RNA content as determined by acridine orange red fluorescence also correlates with histologic grade, but was less discriminatory than the proliferative features. RNA content has been shown in experimental systems to reflect proliferation and cell size, both of which are interrelated.22,41,42 The results of simultaneous DNA and RNA analyses reported herein resemble cytometric observations by other investigators using either light-scatter or Coulter volume analysis coupled with DNA.20,21,23,25-29 In a recent investigation of 220 cases of NHL, Shackney et al noted differences in the relationship of proliferative activity (percentage of cells in S) to Coulter volume among the different lymphoma subgroups.43 Although they are not identical to our "RNA-proliferation maps" (Fig 2), Coulter volume increments seem to correspond to increases in RNA content with progression to more advanced histologic grades. Correlated FCM analysis of DNA, RNA, and cell size should elucidate the independent contributions of these parameters.

In addition, we observed a strong correlation between ds-RE and histologic grade. Although total RNA content and proliferative activity do not allow

| Table 3. Nucleic Acid Cytometry in Malignant Lymphoma: Relationship to Specific Histology |
|-----------------|-------|---------------------------------------------------------------------------------|-------------|--------------------------|
| Working Formulation Category | No. | Aneuploid (%) | Mean (S + G2M)% ± SD | Mean RNA Index ± SD |
| **Low-grade** | | | | |
| Small lymphocytic | 31 | 7 | 4.7 ± 3.0 | 1.07 ± 0.37 |
| Follicular, small cleaved cell | 37 | 21 | 4.4 ± 3.0 | 0.89 ± 0.48 |
| Follicular, mixed small cleaved and large cell | 8 | 37 | 6.6 ± 3.7 | 0.97 ± 0.32 |
| **Intermediate-grade** | | | | |
| Follicular, large cell | 14 | 64 | 8.0 ± 5.0 | 1.30 ± 0.28 |
| Diffuse, small cleaved cell | 18 | 17 | 8.8 ± 2.5 | 1.20 ± 0.44 |
| Diffuse, mixed small and large cell | 7 | 57 | 11.4 ± 7.3 | 1.37 ± 0.69 |
| Diffuse, large cell | 51 | 61 | 15.2 ± 9.4 | 1.96 ± 1.45 |
| **High-grade** | | | | |
| Large cell, immunoblastic | 4 | 100 | 19.0 ± 12.9 | 2.12 ± 0.69 |
| Lymphoblastic | 4 | 50 | 27.7 ± 13.6 | 1.67 ± 0.75 |
| Small noncleaved cell | 7 | 37 | 24.3 ± 5.6 | 1.55 ± 0.58 |
| AIDS-associated lymphoma* | 4 | 0 | 35.0 ± 9.6 | 1.83 ± 0.75 |
| Reactive controls | 27 | 0 | 5.6 ± 2.9 | 0.96 ± 0.28 |

*Three small noncleaved; one diffuse large cell.
diffuse large-cell lymphomas had similar aneuploidy sively from small- to large-cell types. Follicular and activity, and RNA index values increased progres-
correlation with cytometric parameters. For instance,
cell lymphomas.46

There is an association of ds-RNA with tumor
viruses,45 and excess ds-RNA may suggest the patho-
genesis in some lymphomas, since a retrovirus (HTLV) has been implicated in the cause of some T
cell lymphomas.46

Specific morphologic subtypes also showed strong
correlation with cytometric parameters. For instance,
in follicular lymphoma, aneuploidy rate, proliferative
activity, and RNA index values increased progressively from small- to large-cell types. Follicular and
diffuse large-cell lymphomas had similar aneuploidy rates, but the latter group had significantly higher
proliferative activity, which was also true for the
comparison of nodular and diffuse small, cleaved-cell
lymphoma. In the high-grade category, the immuno-
blastic lymphomas had the highest RNA index, a
finding that is in keeping with morphological studies
using methyl green pyronin stains47 and electron
microscopy.48 As expected on the basis of chromosomal
translocation rather than of numeric aberration, small
noncleaved (undifferentiated) lymphomas, both

AIDS- and non-AIDS–related, were more often DNA-
diploid.14,49 The AIDS-related lymphomas showed the
highest proliferative activity of all histologic sub-
groups. The reactive lymphadenopathy seen in homo-
sexual men with AIDS or the AIDS-related complex is
also characterized by a higher proliferative activity
than non–AIDS-reactive controls.50

There is little information concerning the relation-
ship of nucleic acid cytometry and immunophenotypes
in malignant lymphomas. Contrary to observations by
Shackney et al, we did not find a lower aneuploidy rate
in T cell disease as compared with B cell disease.41

The “normal” tissue response in lymph nodes of
patients with malignant lymphomas is poorly under-
stood. In those cases in which aneuploid and diploid
populations could be resolved on the basis of RNA
content, the proliferative activity of the diploid subpop-
ulation was higher than in reactive nodes. This finding
suggests a hyperproliferative response to the presence
of lymphoma in the alleged normal subpopulation.
Although studies addressing this issue are already
feasible in lesions with DNA-aneuploid stemlines,
alternative neoplastic markers such as nucleolar anti-
gen must be identified for study of the remaining
diploid cases.51 Recent technical advances in flow
cytometry and biology should make possible a compre-
hensive interactive analysis of nucleic acid and cell-
surface characteristics to further understanding of the
tumor–host cell interaction.52

For the two groups of patients studied at diagnosis
and relapse without transformation, no differences
between the cytometric variables were noted. This
observation suggests that the development of drug and
radiation resistance is not necessarily associated with
changes in DNA and RNA content, a finding that is in
accord with data in leukemia and solid tumors.53,54 A
transformation from initially indolent lymphoma to a
higher histologic grade was observed in 22% of the
relapse patients. Except for a higher frequency of
aneuploidy, the remaining cytometric features in
transformed lymphoma were similar to those observed
in nodes with intermediate or high grade histology at
presentation. Serial follow-up of individual patients is
required to determine whether the presence of aneu-
plody in indolent lymphoma predicts for subsequent

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<th>Table 4: Grade Prediction Using Acridine-Orange-Derived Parameters in a Discriminant Function Model</th>
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<tr>
<td>Actual Grade</td>
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<tr>
<td>L*</td>
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<td>L</td>
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<td>L</td>
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<tr>
<td>Correct grade prediction, 67%</td>
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<td>(B) With ds-RNA excess (n = 40)</td>
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<td>L</td>
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<tr>
<td>Correct grade prediction, 85%</td>
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<td>*L, low-grade; I, intermediate-grade; H, high-grade histology.</td>
</tr>
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</table>


discrimination between low-grade lymphoma and reactive lymphadenopathy, ds-RE values showed sig-
nificant differences between the two categories. In a
discriminant model, ds-RE alone could correctly clas-
sify 59% of cases and, when combined with acridine
orange parameters, correctly discriminated 85% of
cases. The presence of ds-RNA has previously been
associated with neoplasia but not with proliferation.44
There is an association of ds-RNA with tumor
viruses,45 and excess ds-RNA may suggest the patho-
genesis in some lymphomas, since a retrovirus (HTLV)
has been implicated in the cause of some T

<table>
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<th>Table 5: DNA-Derived Parameters and Remission Induction in Aggressive Lymphoma</th>
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<td>Cytometric Features</td>
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<td>----------------------</td>
</tr>
<tr>
<td>Diploid</td>
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<td>Aneuploid</td>
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<td>(S + G2M) ≤ 15%</td>
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<td>(S + G2M) &gt; 15%</td>
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<td>Data include intermideate- and high-grade histologies. CR, complete remission.</td>
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DNA AND RNA CONTENT IN MALIGNANT LYMPHOMA

histologic transformation. The finding of comparable FCM results in lymph node biopsy and aspirate material suggests that serial fine-needle aspirations may provide representative material for such studies of clonal evolution and/or phenotypic change during a patient’s disease course.

Of equal importance to the differences in cytometric parameters among histologic grades is our observation of considerable heterogeneity within morphologic and immunologic categories of malignant lymphoma. Although the variable natural history of malignant lymphoma has been accounted for in part by morphologic differences, the diversity in nucleic acid features may provide more objective and quantitative criteria for the assessment of prognosis of patients with NHL. Thus, proliferation and ds-RNA excess were the most important features associated with histologic variability, whereas the sensitivity to chemotherapy seemed to be affected by both cytokinetic and ploidy patterns. Further longitudinal studies are necessary to define the relative prognostic roles of morphology, nucleic acid cytometry, and surface phenotyping in NHL.

ACKNOWLEDGMENT

The authors greatly appreciate the editorial advice of Dr Raymond Alexanian and thank Ms Mattie Scott-Thomas for her excellent secretarial assistance.

REFERENCES

31. Reuben JM, Hersh EM, Mansell PW, Newell G, Rossen R,
33. Harris NL, Bhan AK: Distribution of T-cell subsets in follicular and diffuse lymphomas of B-cell type. Am J Pathol 113:177, 1983
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